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PRECURSORS OF INDOXYL

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(Received for publication, November 13, 1943)

The formation of indoxyl by indole and derivatives of indole, and by *o*-nitro and *o*-aminobenzyl compounds, has been studied by Crowdle and Sherwin (1), Houssay *et al.* (2, 3), and by Böhm (4–7) with some divergence in results. The present work adds new compounds to those already studied and reviews the problem.

EXPERIMENTAL

The indoxylogenic action of the substances mentioned in Table I was studied in dogs of both sexes, weighing 7 to 15 kilos. To avoid renal elimination of indoxyl, the dogs were nephrectomized under nembutal anesthesia. Blood indoxyl was determined in samples taken from the carotid artery before and after operation, the average value being selected as the initial concentration of indoxyl. Substances to be tested (0.07 M; 10 to 20 mg. per kilo) were dissolved or suspended in 20 ml. of saline solution and injected into the jugular vein. Indoxyl was determined each hour during the experiment, most of which lasted at least 5 hours.

Increments per hour of plasma indoxyl (mg. per liter) over the initial concentration were averaged; the mean of the values ($I_h = m \pm r$) for a compound was taken as a numerical expression of the indoxylogenic action of the compound. The difference between the average values obtained in different experiments was deemed significant when $\Delta = m_x - m_y \geq 4R$; $R = \sqrt{r_x^2 + r_y^2}$ where m is the mean value and r is the probable error of the mean.

The rôle of the digestive tract and liver in the indoxylogenic action of the compounds under investigation was determined by injecting them into dogs deprived of (1) kidneys, (2) kidneys and digestive tract, (3) kidneys, digestive tract, and liver (abdominally eviscerated). Surgical procedures were carried out as described by Markowitz (8). For extirpation of the liver a Pyrex glass cannula was inserted into the inferior vena cava, fastened with ligatures, and then the suprahepatic veins were cut off.

Plasma indoxyl was determined according to Böhm and Grüner (9) with the Pulfrich photometer; after treatment with Jolles' reagent indoxyl gives cymolindolignone with a maximal extinction at about λ 570 mμ (Filter S-57). After injection of indoleacetic, indolepropionic, or indolepyruvic acid or skatole, treatment of blood plasma filtrates with Jolles' reagent led to the appearance of colors which may interfere in the determination of indoxyl. The position of maximal extinction corresponding to the substances already mentioned is described in Table I as taken with a Hilger spectrograph, Zeiss microphotometer and comparator.

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* Fellow of the Sauberan Foundation.

The error in the indoxyl estimation determined by the substances mentioned is not taken into account except in the case of indoleacetic acid, since the concentration of indoxyl calculated with ϵ (extinction, log I/I_0) corresponding to λ 570 mμ is not significantly different from the concentration observed in the controls.

In the case of indoleacetic acid injection, this was not the case and a correction was made according to the Vierordt method (10) by measuring ϵ in λ 530 mμ and λ 570 mμ and using the following figures of k (specific extinction, gm. $ml.^{-1}$ cm.) determined on indoxyl and indoleacetic solutions: indoxyl, k_{530}, 82.0 ± 1.5; k_{570}, 85.4 ± 1.5; indoleacetic, k_{530}, 25.3 ± 0.8, k_{570}, 16.4 ± 1.1.

TABLE I

Spectral Position of Maximal Absorption of Chloroformic Solution of Pigments Obtained by Treatment with Jolles' Reagent

Substance	Wave-length
	mμ
Skatole	440
Indolealdehyde	481,* 538
Indolepropionic acid	444
Indoleacetic acid	536,* 498
Indolepyruvic "	No selective absorption between 530 mμ and ultraviolet

* The more intense of two maxima.

Tryptophane, indolepropionic acid, indoleethylamine, skatole, indoleacetic acid, and *o*-nitrocinnamic acid were obtained from the Eastman Kodak Company; *o*-nitrophenylpropiolic acid and indolecarbonic acid from Fraenkel Landau; *o*-nitrophenylethanol was prepared according to Sabetay *et al.* (11); *o*-aminophenylethanol according to Bennett and Hafez (12); indolealdehyde, according to Boyd and Robson (13); and indolepyruvic acid, according to Ellinger and Matsuoka (14). The remaining compounds (Table II) were prepared by appropriate methods described in the literature.

Results

As may be deduced from Table II, the compounds which are indoxylogenic in the nephrectomized dog are the following: *o*-aminophenylethanol,

TABLE II

Hourly Increment of Plasma Indoxyl in Dogs after Injection of Various Possible Indoxyl Precursors Following Extirpation of Various Organs

The values represent the hourly increments (mg. per liter of blood) over the experimental period, which lasted in most cases at least 5 hours.

Substance	Extirpated organ					
	Kidneys		Kidneys and digestive tract		Kidneys, digestive tract, and liver	
	No. of experiments	I_h	No. of experiments	I_h	No. of experiments	I_h
o-Nitroacetophenone	3	1.80 ± 0.01	2	0.10 ± 0.07	2	0.00 ± 0.02
o-Aminoacetophenone	2	0.60 ± 0.05				
o-Nitrophenylacetylene	3	3.40 ± 0.42		3.10 ± 0.06	3	3.00 ± 0.24
o-Aminophenylacetylene	2	0.70 ± 0 12				
o-Nitrophenylethanol	5	3.90 ± 0.40	3	0.30 ± 0.08		
o-Aminophenylethanol	3	18.00 ± 0.30	3	14.00 ± 2.70	3	0.00 ± 0.04
o-Nitrophenylacetaldehyde	3	1.80 ± 0.03	3	0.16 ± 0.04		
o-Nitrophenylacetic acid	3	0.56 ± 0.02				
o-Aminophenylacetic acid	3	1.00 ± 0.29				
o-Nitrocinnamic acid	3	0.80 ± 0.06				
o-Aminocinnamic acid	2	0.30 ± 0.05				
o-Nitrophenylpropiolic acid	2	16.80 ± 0.96	3	13.30 ± 0.48	3	4.00 ± 0.69
o-Aminophenylpropiolic acid	2	0.52 ± 0.16				
o-Nitrophenylpyruvic acid	3	0.67 ± 0.09				
o-Nitrobenzoylacetic acid	3	1.70 ± 0.05	3	1.20 ± 0.12	3	0.07 ± 0.02
Indole	4	14.30 ± 0.90	3	12.40 ± 0.14	5	4.40 ± 0.14
Skatole	3	0.77 ± 0.14				
Indolealdehyde	3	1.26 ± 0.08				
Indolecarbonic acid	3	0.83 ± 0.10				
Indoleethylamine	3	0.62 ± 0.30				
Indoleacetic acid	2	0.61 ± 0.01				
Indolepropionic acid	4	0.77 ± 0.14				
Indolepyruvic acid	3	0.58 ± 0.05				
Tryptophane	3	0.52 ± 0.04				
Controls	12	0.70 ± 0.03	10	0.13 ± 0.05	6	0.00 ± 0.01

o-nitrophenylpropiolic acid, indole, *o*-nitrophenylethanol, *o*-nitrophenylacetylene, *o*-nitroacetophenone, *o*-nitrophenylacetaldehyde, *o*-nitrobenzoylacetic acid, and indolealdehyde. The I_k of *o*-aminophenylacetic acid and *o*-nitrocinnamic acid was greater than that corresponding to the controls, but as $\Delta < 4R$, it was not significant.

Indole, *o*-aminophenylethanol, *o*-nitrophenylpropiolic acid, *o*-nitrophenylacetylene, and *o*-nitrobenzoylacetic acid were transformed into indoxyl in nephrectomized dogs and dogs deprived of the digestive tract. In abdominally eviscerated dogs, only indole, *o*-nitrophenylacetylene, and *o*-nitrophenylpropiolic acid produced indoxyl.

DISCUSSION

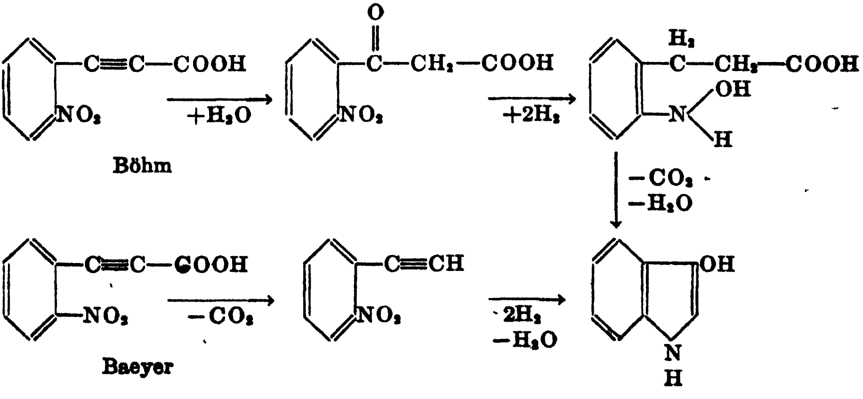
Among the indolic compounds studied only indole and to a very small extent indolealdehyde produce indoxyl This is in harmony with Böhm's (4) results; contrary observations by other authors (3) might be explained on the basis of error in the colorimetric determination of indoxyl due to the presence of non-specific pigments. If mammals can produce indoxyl other than that of intestinal origin (2, 3), its formation could result not by loss of the side chain of tryptophane but rather through rupture of the pyrrole nucleus of this amino acid with formation of *o*-aminobenzene derivatives, as maintained by Krebs *et al.* (15) in regard to the formation of indole by *Bacillus coli*. The intense indoxylogenic action of *o*-aminophenylethanol in the dog lends experimental support for this hypothesis.

The rôle of the liver in the indole-indoxyl transformation has been the subject of much discussion. According to Gautier, Hervieux, and Laroche (cited by Houssay (3)) the liver is presumed to be the only organ able to effect this transformation. Extrahepatic oxidation of indole to indoxyl was supported with the aid of inconclusive experiments by Gaetani, Pinelli, and Pugioni (*cf.* (16)), and by García and Nacle (17), Macchia (18), and by Barac (19). In the present experiments the extirpation of the liver in the manner described allows the total elimination of this organ, and the method for determination of indoxyl is accurate enough to assure an extrahepatic oxidation of indole corresponding to 30 per cent of the total observed in normal dogs. In the toad *Bufo arenarum* Hensel, in which no indoxyl is normally detectable in the blood, extrahepatic oxidation of indole is equal to 50 per cent of that observed in normal animals; so that the enzyme systems concerned in the oxidation of indole seem to be but little localized (16).

o-Aminophenylethanol is intensely indoxylogenic in the dog. The possibility of transformation of *o*-aminobenzene derivatives into indoxyl had been denied by Böhm (6) on the ground of negative results with *o*-aminoacetophenone, *o*-aminophenylpropiolic acid, and *o*-aminocinnamic acid. These results can be accounted for by the inability of animal tissues to produce in the β-carbon of the side chain a RCHO group which can react with the amino group, as happens with *o*-aminophenylethanol (15).

The indoxylogenic action of *o*-nitrophenylpropiolic acid is well known. Of the mechanisms proposed for this reaction by Crowdle and Sherwin (1) and Böhm (6), that proposed by Böhm seems to agree better with the observed data. However, a number of considerations support the belief that the rôle of the intermediates suggested is indeed limited. It may be noted from Table II that the indoxylogenic action of *o*-nitrobenzoylacetic acid in dogs whose kidneys and digestive tracts have been removed is significantly less than that of *o*-nitrophenylpropiolic acid, while theoretically it should at least equal it. In abdominally eviscerated dogs *o*-nitrobenzoylacetic acid does not produce indoxyl; so that it cannot be considered in this case an intermediate in the transformation of *o*-nitrophenylpropiolic acid into indoxyl; finally the indolecarbonic acid tacitly considered as an intermediate in this transformation has no indoxylogenic properties in any case.

In abdominally eviscerated dogs only *o*-nitrophenylacetylene produced indoxyl among all the possible intermediates considered and to an extent not significantly different from *o*-nitrophenylpropiolic acid. The fact allows one to suppose that *o*-nitrophenylpropiolic acid is partly decarboxylated, producing *o*-nitrophenylacetylene; this substance is then transformed into indoxyl. This latter transformation seems to be performed by means of an internal oxidation-reduction reaction, as originally proposed by Baeyer (20). In dogs whose kidneys and digestive tracts were removed, or in abdominally eviscerated dogs, none of the compounds which might be considered possible intermediates in the transformation of *o*-nitrophenylacetylene into indoxyl had any indoxylogenic properties. This does not rule out the possibility that in normal or nephrectomized dogs the reaction of *o*-nitrophenylpropiolic acid into indoxyl may be carried out in two ways: that proposed by Baeyer (20) and that described by Böhm (4–7) (see the accompanying schemes).



SUMMARY

The indoxylogenic action of various indole derivatives and *o*-nitro and *o*-amino compounds was studied in the dog. Indole and to a very slight extent indolealdehyde are the only indole compounds producing indoxyl. Extrahepatic oxidation of indole is equal to 30 per cent of that observed in intact dogs. Among the *o*-aminobenzene derivatives studied only *o*-aminophenylethanol produced indoxyl.

o-Nitrophenylpropiolic acid and *o*-nitrophenylacetylene produce indoxyl in abdominally eviscerated dogs. *o*-Nitrophenylacetylene can be considered an important intermediate in the transformation of indoxyl from *o*-nitrophenylpropiolic acid. The presence of the liver is essential for the indoxylogenic action of *o*-nitrobenzoylacetic acid and *o*-aminophenylethanol; the digestive tract is necessary for the indoxylogenic action of *o*-nitrophenylethanol, *o*-nitroacetophenone, and *o*-nitrophenylacetaldehyde.

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BIBLIOGRAPHY

1. Crowdle, J. H., and Sherwin, C. P., *J. Biol. Chem.*, **55**, 15 (1923).
2. Houssay, B. A., *Am. J. Med. Sc.*, **192**, 615 (1936).
3. Houssay, B. A., Deulofeu, V., and Mazzocco, P., *Compt. rend. Soc. biol.*, **119**, 875 (1935); *Rev. Soc. argent. biol.*, **11**, 7, 18 (1935).
4. Böhm, F., *Biochem. Z.*, **290**, 137 (1937).
5. Böhm, F., *Z. physiol. Chem.*, **255**, 205 (1938); **260**, 1 (1939).
6. Böhm, F., *Z. physiol. Chem.*, **261**, 35 (1939).
7. Böhm, F., *Z. physiol. Chem.*, **265**, 210 (1940).
8. Markowitz, J., Textbook of experimental surgery, London, 491 (1937).
9. Böhm, F., and Grüner, G., *Klin. Wochschr.*, **15**, 450 (1936).
10. Heilmeyer, L., Spectrophotometry in medicine, London, 7 (1943).
11. Sabetay, S., Bleger, J., and de Lestrange, Y., *Bull Soc. chim.*, **49**, 3 (1931).
12. Bennett, G. M., and Hafez, M. M., *J. Chem. Soc.*, 287 (1941).
13. Boyd, W. J., and Robson, W., *Biochem J.*, **29**, 557 (1935).
14. Ellinger, A., and Matsuoka, Z., *Z. physiol. Chem.*, **109**, 259 (1920).
15. Krebs, H. A., Hafez, M. M., and Eggleston, L. W., *Biochem. J.*, **36**, 306 (1942).
16. Stoppani, A. O. M., *Rev. Soc. argent. biol.*, **19**, 435 (1943).
17. García Blanco, J., and Nacle, J., *An. Soc. españ. fis. y quim.*, **33**, 105 (1935).
18. Macchia, E., *Boll. Soc. ital. biol. sper.*, **13**, 717 (1935).
19. Barac, G., *Compt. rend. Soc. biol.*, **126**, 1239 (1937); **132**, 41 (1939); *Arch. internat. physiol.*, **50**, 1 (1940).
20. Baeyer, A., *Ber. chem. Ges.*, **13**, 2259 (1880).

THE PROTEIN-FORMALDEHYDE REACTION

III. THE EFFECT OF TEMPERATURE AND DENATURATION

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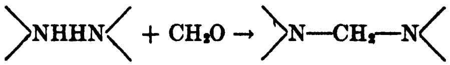
In Papers I (5) and II (8), the reaction of formaldehyde with collagen and wool keratin was discussed. It was shown that for formaldehyde fixation by collagen from a 1 per cent formaldehyde solution, the fixation increased almost as a straight line function from pH 1.0 to pH 6.5 (the point of zero acid or base fixation). At pH 6.5, there was a definite break in the fixation curve, the fixed formaldehyde increasing sharply to 0.5 milliequivalent and then remaining essentially constant from pH 7.0 to 9.5. At pH 9.5 another break appears, the fixed formaldehyde increasing sharply at this point and continuing up to pH 11.5, at which point approximately 0.81 milliequivalent of aldehyde is bound. Interpreting these data, Theis postulated that in the pH zone 1.0 to 6.5 the formaldehyde bridged adjacent polypeptide chains of the collagen by means of weakly basic imino groups in juxtaposition



In the pH range 7.0 to 9·5, the reaction was believed to be with the imidazole group of histidine and at pH values greater than 9.5 with the ϵ-amino group of lysine. It was further pointed out that at pH values more acid than the isoionic point, the formaldehyde would not bind with the basic groups of lysine and arginine, since these would be expected to exist in the charged ionic form and thus the electronic pair of the nitrogen atom would not be available for aldehyde fixation.

In a previous paper, Theis and Jacoby (7) discussed the acid- and base-binding capacity of heat-denatured collagen and showed that denaturation shifted the point of zero acid or base fixation to a more alkaline one. Since no literature reference could be found relative to the binding of formaldehyde by heat-denatured proteins, the following data were obtained.

EXPERIMENTAL

Specially prepared collagen material (6) was used for these experiments. The preparation of this collagen has been previously described. 2 gm. samples of the collagen were placed in water at 70° for 1 minute, thus allowing instantaneous shrinkage and denaturation to take place. The denatured samples were then placed in bottles together with 200 ml. of 0.1 N KCl solution made 1 per cent with respect to formaldehyde, after which the samples were adjusted to a series of definite hydrogen ion concentrations with either hydrochloric acid or sodium hydroxide. The range covered was from pH 1.0 to 12.5. The bottles were placed in a thermostat maintained at 20° for 72 hours. At stated intervals, the samples were agitated in order to promote equilibrium. After the 72 hour period, the pH at equilibrium was determined by means of a Beckman glass electrode assembly; the collagen material was removed and pressed several times between blotting paper at 10,000 pounds per sq. in. It has been previously shown that for all practical purposes such pressure removes free water together with any free electrolyte (6). We, therefore, have assumed that all free formaldehyde is correspondingly removed, leaving behind only that which is firmly bound to the collagen itself. After being pressed, the collagen-formaldehyde was allowed to dry in air, and then was ground in a small Wiley mill to a 60 mesh powder. The material was then ready for analysis for nitrogen, for bound acid or base, and for fixed formaldehyde. These methods have been previously described (6, 5).

Fig. 1 shows the data obtained. The series of curves may be interpreted as follows: Curve A′ represents the regular acid and base fixation for the native collagen used, while Curve A represents that for the formaldehyde-treated collagen. Curve B represents the formaldehyde fixed by collagen from a 1 per cent solution of formaldehyde. These curves were fully described and discussed in Paper I. Curve C represents the titration curve of formaldehyde-treated heat-denatured collagen. The trend of this curve shows (1) a higher maximum acid fixation than that obtaining for undenatured collagen, (2) a greater acid fixation in the pH zone 1.0 to 8.5, (3) a shift of the isoionic point to a more alkaline reaction, and (4) a lessened base fixation in the zone pH 8.5 to 12.0. Curve D represents formaldehyde fixed by the heat-denatured collagen over a wide pH range. These data indicate (1) greater formaldehyde fixation over the entire pH range, (2) a break in fixation at pH 3.0, another at pH 4.0, and a third at pH 7.5, (3) a decided shift in the plateau zone to a more acid one, and (4) a much greater formaldehyde fixation in the alkaline region.

In another series of experiments, the effect of reaction temperature was studied. The experimental procedure was identical to that already described, with the exception that native and not heat-denatured collagen was used. The temperatures employed were 2.5° and 60°. In order not to cause heat denaturation at 60°, the samples were allowed to react with the formaldehyde for 24 hours at room temperature, after which they were subjected to a 60° temperature for an additional 48 hours. Even with such precautions, pH values lower than 4.3 and greater than 10.4 caused hydrolysis and denaturation and the data could not be used.

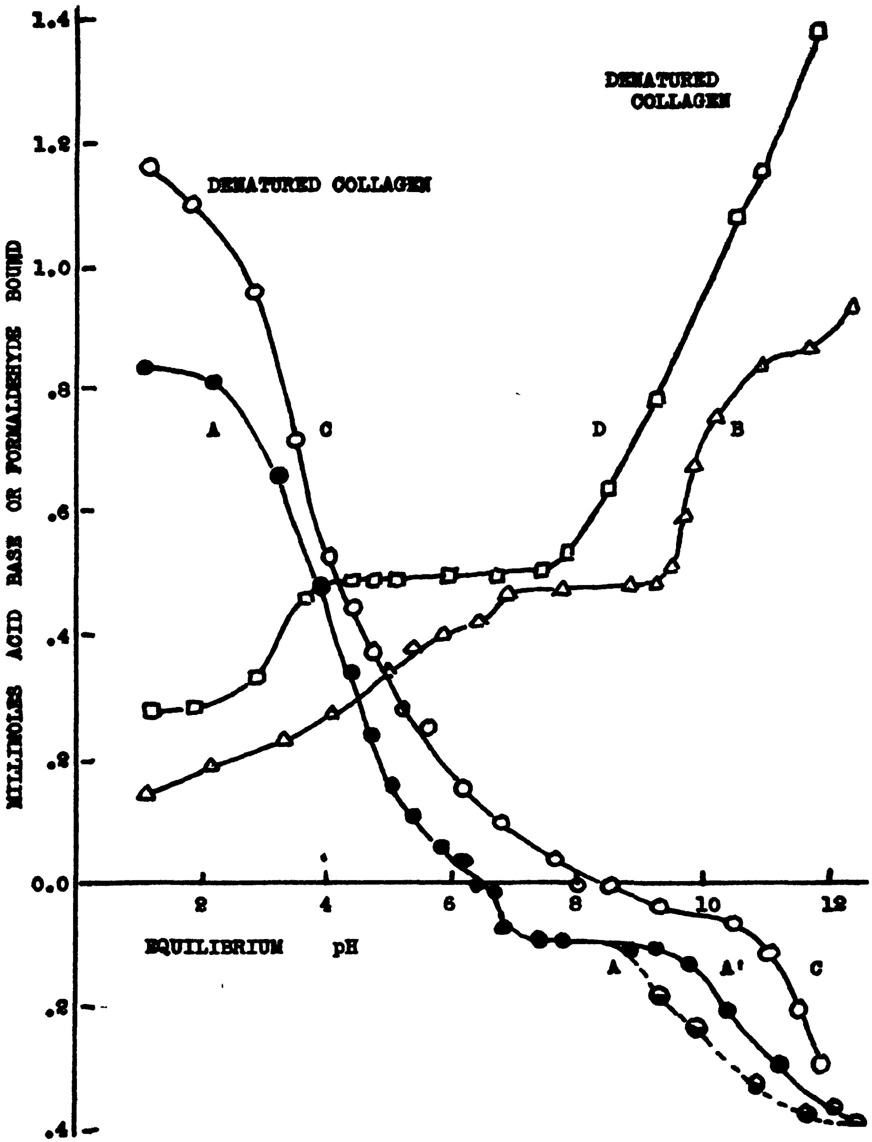


FIG. 1. Showing effect of heat denaturation upon the formaldehyde fixation of collagen. The curves are explained in the text.

The observations are shown in Fig. 2 and may be interpreted as follows: Curve A represents the acid and base bound by the collagen at 2.5° and indicates in the main approximately the same trend as that shown for the collagen-formaldehyde titration curve at 20°. Curve C represents the acid and base bound by the collagen at 60° and shows (1) in the pH range 4.3 to 7.1 a greater acid fixation, (2) a slight shift in the isoionic point to a more alkaline point, and (3) a lessened base fixation in the zone pH 7.1 to 10.4. Curve B represents the formaldehyde fixation by collagen at 2.5° and shows (1) in the acid zone pH 2.0 to 6.0 a lessened aldehyde fixation than that obtaining at 20° and about the same fixation at pH values greater than pH 7.0, and (2) striking points of inflection at pH 6.5 and pH 9.6. Curve D represents the formaldehyde fixation obtaining at 60° in the pH range 4.2 to 10.4 and shows (1) a greater over-all formaldehyde fixation, (2) a shift to the more acid range of the plateau zone, and (3) definite points of inflection at pH 6.0 and pH 8.1.

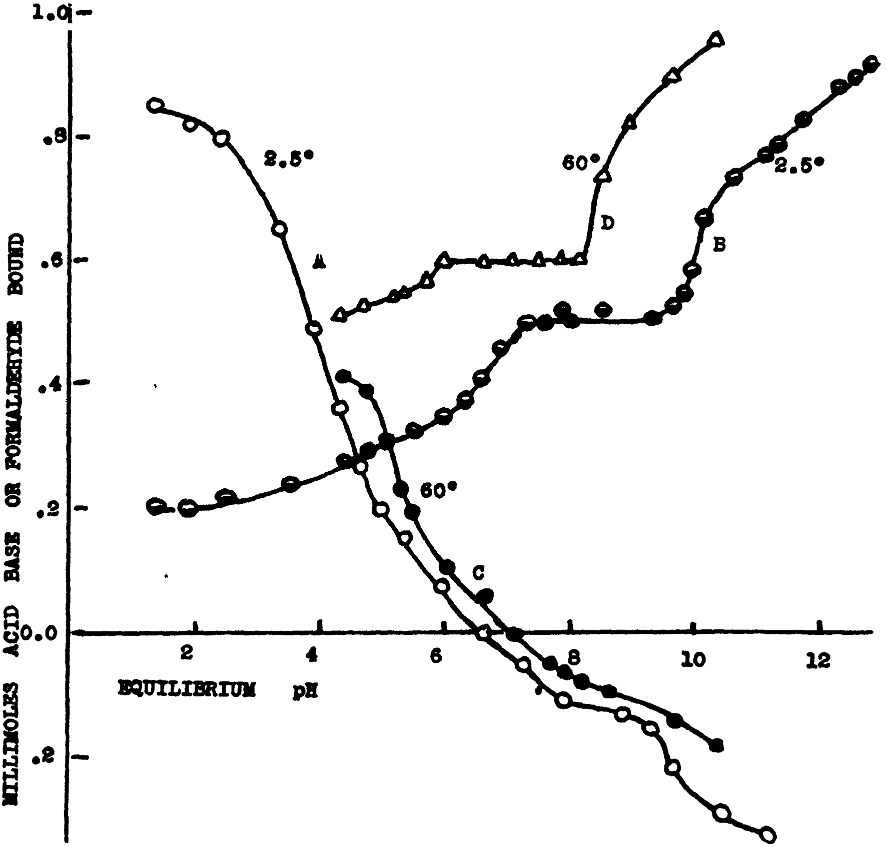


Fig. 2. Showing effect of temperature of reaction upon the formaldehyde fixation of collagen.

Salcedo and Highberger (4) investigated the effect of the drying at 100° of the collagen-formaldehyde compound and came to the conclusion that, regardless of the formaldehyde concentration originally used, the final compound obtained after such drying corresponded to a ratio of 1 molecule of formaldehyde for every free amino group.

In another series of experiments, the effect of the drying of the collagen-formaldehyde compound was investigated. Similar technique to that already described was employed. In this case, however, native collagen was treated with 1 and with 5 per cent formaldehyde solutions for 72 hours at 20°. After treatment, the collagen was pressed and that treated with 5 per cent formaldehyde was divided in half, the one-half being used for the regular analysis while the other half was dried *in vacuo* at 100° for 24 hours before analysis. The collagen-formaldehyde compound formed through the reaction with the 1 per cent formaldehyde solution was not dried. Fig. 3 shows the data obtained and may be interpreted as follows: Curve A represents the titration curve of the dry heated collagen-formaldehyde compound and shows but little change from that of any of the titration curves in the acid zone. It does show some little change from that of the collagen-formaldehyde compound formed regularly through reaction with a 5 per cent formaldehyde solution, in the pH range 6.8 to 9.0. Curves B and C represent formaldehyde fixation from a 1 and 5 per cent formaldehyde solution respectively and are identical to those given in Paper I. Curve D represents the formaldehyde fixed by the collagen from a 5 per cent formaldehyde solution and then heated *in vacuo* at 100° for 24 hours. These data show (1) less fixed formaldehyde after heating, but more than that obtaining in the collagen-formaldehyde compound formed from the 1 per cent formaldehyde solution, (2) a decided shift to the more acid region of the broad plateau zone, and (3) a greater removal of formaldehyde in the pH range 5.0 to 11.0.

DISCUSSION

Theis and Jacoby (7) found that heat-denatured collagen had about the same maximum acid- and base-binding capacity as that of the native collagen, but that the point of zero combination of the denatured collagen had shifted to a more alkaline point. The results of this investigation were contrary to the findings of Loughlin (1) but in agreement with the data of Michaelis and Davidsohn (2) and Mirsky and Pauling (3).

For the supercontracted collagen, Theis and Jacoby postulated a change in molecular structure since it is highly probable that, during the collapse of the collagen structure upon itself during the heat denaturation, there is a breakdown of existing linkages with the reforming of others.

Fig. 1 shows rather strikingly that heat denaturation affects the aldehyde fixation in just as drastic a manner as it affects the acid and base fixation.

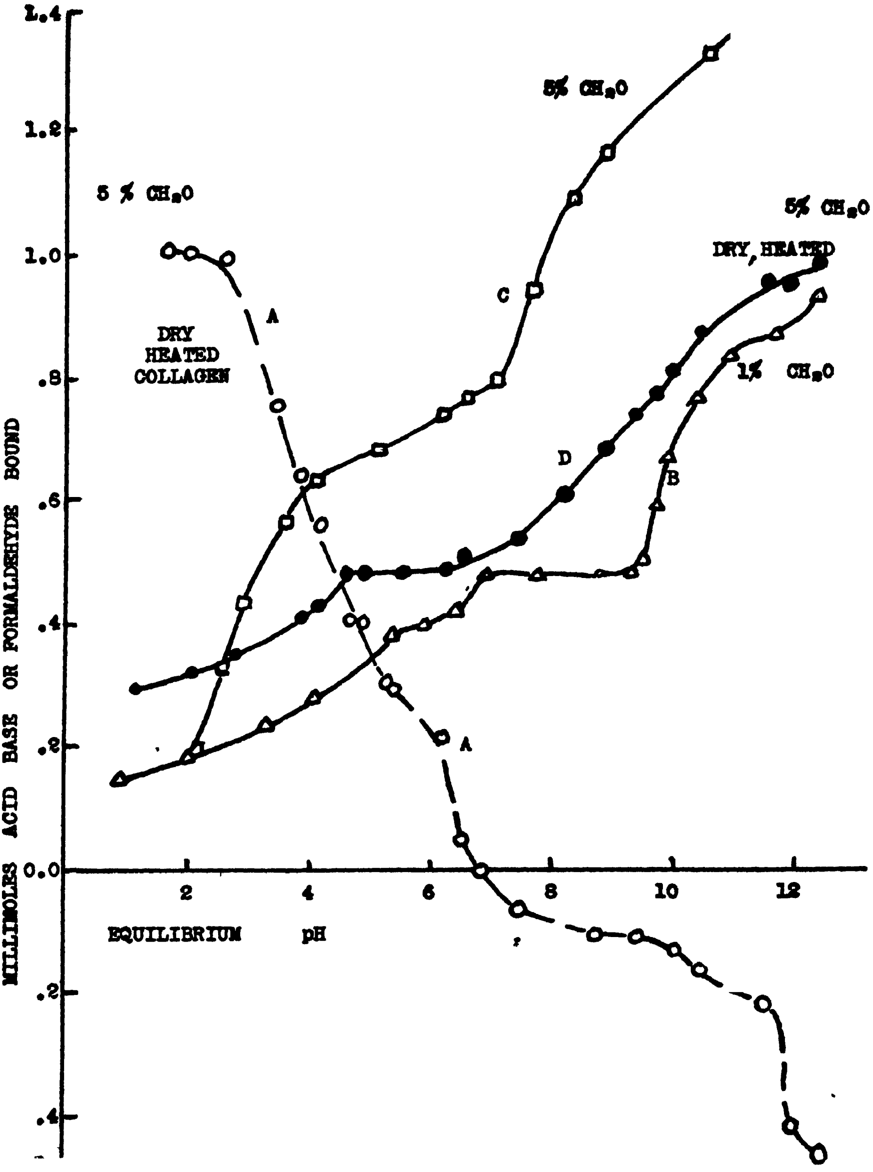
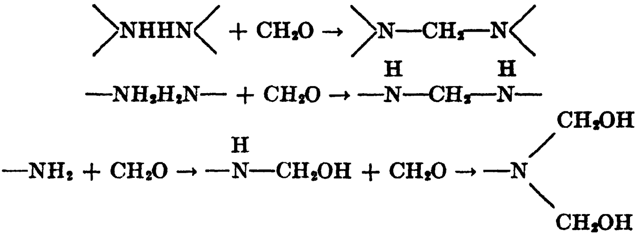


Fig. 3. Showing the effect of heating after drying upon formaldehyde fixation

In this case, however, this difference manifests itself in (1) giving a real increase in aldehyde fixation over the entire pH range, and (2) in a complete shift of the fixation curve to a more acid zone.

Theis believes that normal collagen binds formaldehyde in several distinctly different ways. These are (1) bridging of polypeptide chains by means of weakly basic imino groups in juxtaposition and occurring over the whole pH range and accounting in the main for the increased structural resistance of the collagen-formaldehyde compound, (2) binding of polypeptide chains by means of the imidazole and ϵ-amino groups of histidine and lysine at pH values greater than 6.5 and 9.5 respectively, and (3) binding with the ϵ-amino groups of lysine in methylol fashion. Thus these reactions might be pictured:



(1) $$>NHHN< + CH_2O \rightarrow >N-CH_2-N<$$

(2) $$-NH_2H_2N- + CH_2O \rightarrow -\overset{H}{N}-CH_2-\overset{H}{N}-$$

(3) $$-NH_2 + CH_2O \rightarrow -\overset{H}{N}-CH_2OH + CH_2O \rightarrow -N{<}^{CH_2OH}_{CH_2OH}$$

Since the acid fixation capacity of the collagen is in no way affected by the formaldehyde reaction in the acid zone, obviously reactions (2) and (3) do not occur in this zone. However, as the pH value increases and the charged NH_3^+ groups are dissociated, reactions (2) and (3) occur.

With the internal collapse of the collagen structure upon itself during the heat denaturation, a change in dipolar ion configuration might well have occurred, resulting in the conversion of certain electrovalent salt linkages to coordinate ones. Further, the structural breakdown might well bring into juxtaposition more of the weakly basic imino groups of adjacent polypeptide, chains thus resulting in a further bridging and increased aldehyde fixation. Such an interpretation would account for the increased fixation noted in the pH range 2.0 to 7.0. In the pH range 7.0 to 7.5 the aldehyde fixed is approximately the same for both the native and denatured collagen. At pH values greater than 7.5, the curve rises rapidly and supposedly the ϵ-amino groups of lysine begin to react.

Fig. 2 shows the increased aldehyde fixation occurring through increased reaction temperature. Though the precaution of pretreatment at room temperature was used, it is quite possible that some heat denaturation took place up to pH 7.0 and this may well account for the slight shift in the isoionic point. The increase in aldehyde fixation with increasing temperature is normal and would be expected. Increased temperature would act in a similar manner to a great excess of formaldehyde, causing a greater activity.

Curve D of Fig. 3 shows that dry heat will volatilize an appreciable amount of formaldehyde when excessive amounts of aldehyde are fixed. This fact is especially evident in the alkaline zone. Dry heating of the collagen-formaldehyde compound at 100° causes not only a loss in aldehyde but a shift in the broad plateau region of the curve. It has been shown in a previous paper (5) that aldehyde fixation in the alkaline zone was approximately the same when 0.25, 0.50, and 1.0 per cent formaldehyde solutions were used. The use of higher concentrations of formaldehyde gave a greatly increased fixation in this zone. It therefore appears that if the collagen-formaldehyde compound is heated, it tends to approach those fixation values obtaining for the compound produced by the 1 per cent formaldehyde solution.

Curve A of Fig. 3 shows that the isoionic point of the collagen-formaldehyde compound remains essentially the same as that for the native collagen.

The data for formaldehyde fixation appear to indicate again that (1) in the acid zone aldehyde fixes with the weakly basic imino groups of the collagen, (2) in the isoionic zone aldehyde binds with the imidazole group of histidine, (3) in the zone having a pH greater than 8.0 the binding is with the ϵ-amino group of lysine, and (4) the guanidino group of arginine does not react with formaldehyde under the existing experimental conditions.

Denaturation of collagen brought about by supercontraction of the structure appears to cause the formation of new active centers, especially in the isionic region, which in turn cause a change in aldehyde-binding capacity of the heat-denatured material. Such a change in formaldehyde fixation is readily noticeable in Curve D of Figs. 1 to 3.

SUMMARY

The effect of heat denaturation of collagen upon formaldehyde fixation has been investigated. This study shows (1) heat denaturation increases the aldehyde fixation over the entire pH range, (2) denaturation causes a shift in the broad plateau zone of the aldehyde fixation curves, and (3) the earlier interpretations relative to formaldehyde fixation with collagen are substantiated.

BIBLIOGRAPHY

1. Louglin, W. J., *Biochem. J.*, **27**, 97 (1933).
2. Michaelis, L., and Davidsohn, D., *Biochem. Z.*, **33**, 456 (1911).
3. Mirsky, A. E., and Pauling, L., *Proc. Nat. Acad. Sc.*, **22**, 439 (1936).
4. Salcedo, I. S., and Highberger, J. H., *J. Am. Leather Chem. Assn.*, **36**, 271 (1941).
5. Theis, E. R., *J. Biol. Chem.*, **154**, 87 (1944).
6. Theis, E. R., and Jacoby, T. F., *J. Biol. Chem.*, **146**, 163 (1942).
7. Theis, E. R., and Jacoby, T. F., *J. Biol. Chem.*, **148**, 105 (1943).
8. Theis, E. R., and Lams, M. M., *J. Biol. Chem.*, **154**, 99 (1944).

THE PROTEIN-FORMALDEHYDE REACTION

IV. DENATURATION BY DEAMINIZATION OF COLLAGEN

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In previous papers (8, 7) we have discussed the heat denaturation of collagen and have pointed out that the supercontraction of the collagen upon itself changes the acid- and base-binding capacity in the isoionic zone and increases the formaldehyde fixation over the pH range studied.

Investigations over a great many years indicate that in the case of proteins nitrous acid affects in the main only the ε-amino groups of lysine residues. Wiley and Lewis (12) and Stendel and Schumann (5) have confirmed the destruction of lysine during nitrous acid treatment. Wiley and Lewis state, however, that during such treatment arginine remains unaffected. Stendel and Schumann take issue with this and claim the partial destruction of the arginine of the protein. Both Wiley and Lewis and Stendel and Schumann state that some 50 per cent destruction of histidine occurs during the nitrous acid treatment. Prideaux and Woods (4) maintain that the deaminization must be done in the cold; otherwise certain side reactions ensue which may result in unaccounted for changes. These investigators state that even at room temperatures nitroso groups are formed.

Meunier and Schweikert (3) investigated the properties of deaminized collagen. Their data showed that deaminization caused a decrease in formaldehyde fixation, that such difference was greater at pH values less than 7.0, and that in no case was the ability to fix formaldehyde lost entirely. Highberger and Retzsch (2) studied the formaldehyde-binding capacity of deaminized collagen. They state that their formaldehyde fixation curve for deaminized collagen is very similar to that for native collagen, that the formaldehyde bound is lower in all cases, that the slope of the curve for the deaminized material is much less steep below pH 7.0, while above pH 8.0 the slope of the curve is about the same as that for normal collagen. Highberger and Retzsch claim that the low aldehyde-binding capacity of deaminized collagen at pH values less than 8.0 is due to residual lysine residues and that the increased binding at pH values greater than 8.5 is due to the reaction of the guanidino groups of arginine with formaldehyde.

Bowes and Pleass (1) investigated the reaction of formaldehyde with deaminized collagen and in the main came to the same general conclusions as those of Highberger and Retzsch.

Deaminization with nitrous acid must destroy some few of the electrovalent salt linkages that are assumed to exist between adjacent polypeptide chains. Such changes would then evidence themselves in the breakdown of the internal cohesional forces of the collagen. This breakdown of salt linkages—$NH_3^{+}{}^{-}OOC$— should be especially marked in the so called pH stability zone or in the isoionic zone. Nitrous acid treatment can certainly be stated to be a denaturing one and the data given in the paper will deal with the formaldehyde fixation of collagen which has been denatured through deaminization.

EXPERIMENTAL

Specially prepared collagen was deaminized according to the method outlined by Highberger and Retzsch (2) and Thomas and Foster (11). The general methods employed in the present investigation are identical with those described in two previous papers (7, 6) and therefore will not be further discussed.

Figs. 1 and 2 show the data so obtained and may be interpreted as follows:

Curve A (Fig. 1) represents the swelling of the normal native collagen over the pH range 1.0 to 12.0 and shows minimum values at approximately pH 5.2 and pH 8.0.

Curve B represents the swelling observed for native collagen treated with formaldehyde. These data indicate that formaldehyde treatment represses the swelling of collagen in the pH range 1.0 to 5.5 and in the pH range 8.0 to 12.0. In the zone pH 5.5 to 8.0 swelling is essentially that obtaining for normal collagen. The swelling curve for the collagen-formaldehyde compound shows a slight shift of the minimum value from pH 5.2 to 4.9, a slight shift of the maximum from pH 7.3 to 6.5, and a flat plateau in the pH zone8.0 to 10.5 in place of the sharp minimum obtaining for native collagen.

Curve C represents the swelling of the deaminized collagen and shows a minimum value at pH 4.2 and a plateau zone in the pH range 6.5 to 8.5. The swelling of deaminized collagen is less than that of either the native or formaldehyde-treated collagen in the pH range 1.0 to 3.6. Curve C shows a decided shift of the minimum value from pH 5.2 to 4.2.

Curve D represents the swelling of the formaldehyde-treated deaminized collagen. These data indicate a minimum value at pH 5.3 and then a steady and continuous increase of swelling with increasing pH value. Curve D shows a repression of swelling from that indicated by Curve C over the entire pH range and a drastic reduction from the native and formaldehyde-treated native collagen in the pH range 1.0 to 4.5.

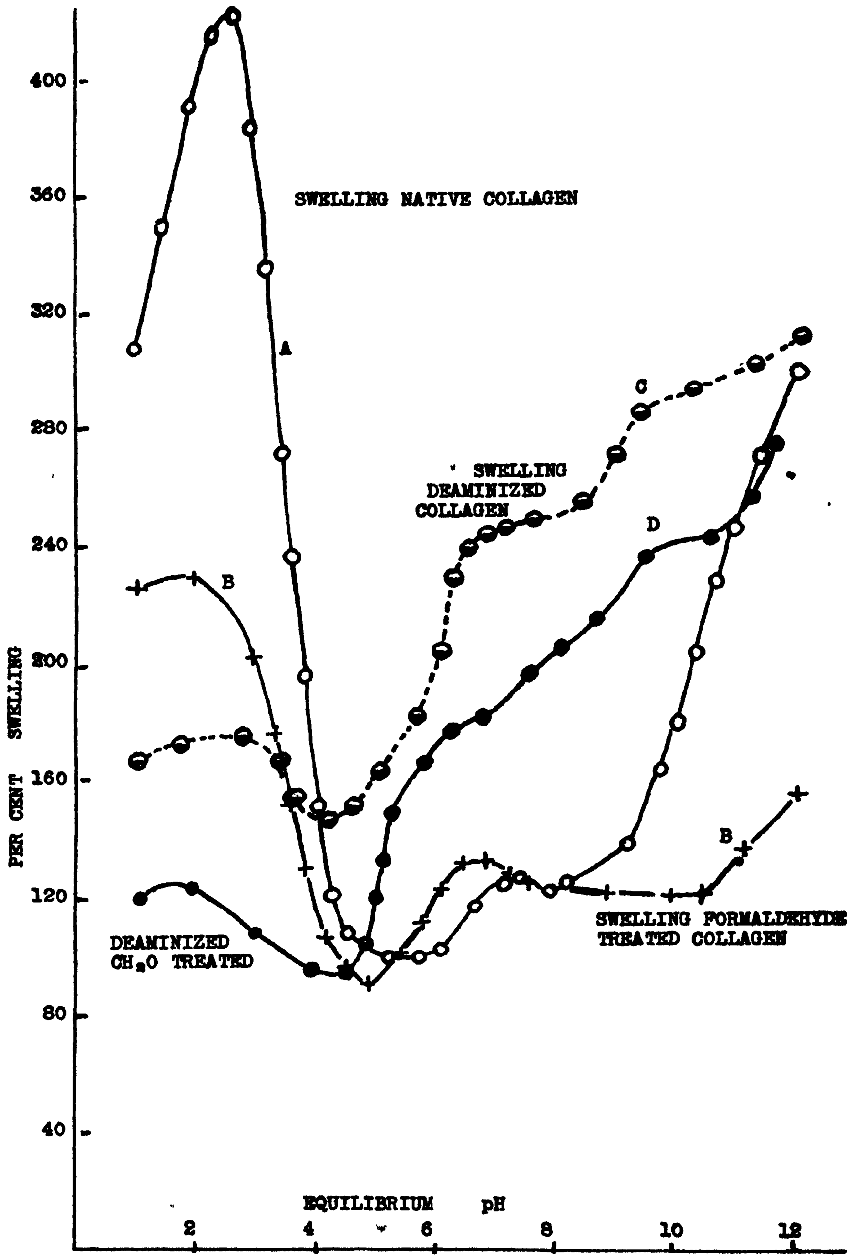

Fig 1. Showing swelling of native collagen (Curve A), formaldehyde-treated collagen (Curve B), deaminized collagen (Curve C), and formaldehyde-treated deaminized collagen (Curve D)

Curves C and D show strikingly the effect of denaturation by deaminization in that swelling is greatly increased over that of native collagen at pH

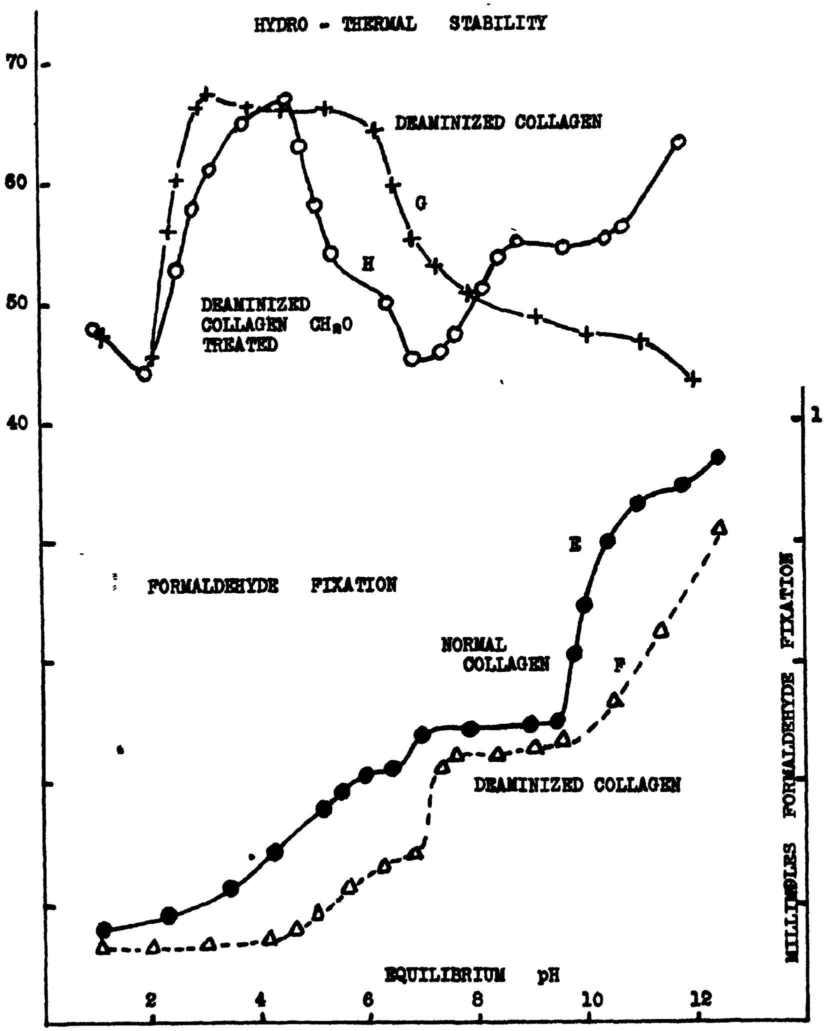

Fig. 2 Showing hydrothermal stability and formaldehyde-binding capacity of deaminized collagen.

values greater than 5.0. Thomas and Foster (11) obtained similar data.

Curve E (Fig. 2) represents data for formaldehyde fixation by native collagen and has been previously discussed in detail (9).

Curve F represents the formaldehyde fixed by deaminized collagen. These data are somewhat different from the observations of Highberger and Retzsch in that (1) no drastic reduction of formaldehyde fixation is noted in pH zone 7.0 to 9.5, and (2) the general trend of the curve in the zone pH 1.0 to 7.0 shows a less marked reduction in formaldehyde fixation.

Curve G indicates the shrinkage temperature for deaminized collagen over the pH range noted. Whereas native collagen has a shrinkage temperature of approximately 58° over the pH range 4.0 to 9.0, the curve for deaminized collagen shows a plateau in the zone pH 3.0 to 6.0 with striking decreases on either end of the plateau. In addition the nitrous acid treatment has materially increased the shrinkage temperature to a value of 66.5° at pH 3.0. The striking decrease in shrinkage temperature in the range pH 6.0 to 8.0 is unusual for collagen.

Curve H represents the shrinkage temperature of the deaminized collagen-formaldehyde compounds formed. This curve is still more unusual for collagen compounds in that it shows a maximum value of 66° at pH 3.5. There is a drastic decrease in shrinkage temperature at pH values either lower or higher than this point. However, the shrinkage temperature again increases at pH values greater than pH 7.0, indicative of another reaction between the deaminized collagen and formaldehyde.

DISCUSSION

Highberger and Retzsch (2), in interpretation of their data, claim in the case of deaminized collagen that the formaldehyde binds with the residual ϵ-amino groups of lysine at pH values less than 7.0, and that at pH values greater than 8.5 the reaction is entirely with the guanidino groups of the arginine residues. Their data show, for deaminized collagen, a drastic reduction in aldehyde fixation as compared with that for normal collagen. This decrease holds for the pH range studied, as can be seen from Table I.

The data of Highberger and Retzsch were obtained through analysis of the collagen-formaldehyde compound after it had been thoroughly washed with water. The writer believes that this particular leaching technique reverses to a large extent the collagen-formaldehyde equilibrium, and as a consequence the total fixed formaldehyde is smaller than that actually obtaining at true equilibrium. The pressing technique, as used by the writer (6), removes only that formaldehyde which is free and thus leaves behind that which is actually fixed.

A study of the various curves in Figs. 1 and 2 indicates that the nitrous acid has materially altered the internal structure of the collagen. Curves C and G show definitely that in the isoionic zone, pH 6.0 to 8.0, there is a real breakdown of the internal cohesional forces. This is evident in the high alkaline swelling and the drastic decrease in the shrinkage temperature of the deaminized collagen. Curves D and H show the changes that take place relative to swelling and shrinkage temperature upon treatment of the deaminized collagen with formaldehyde. The results indicate that in spite of formaldehyde treatment, no strengthening of the internal cohesional forces occurs in the pH range 3.0 to 5.0. At pH values 5.0 to 8.0 an actual decrease in shrinkage temperature is noted. At pH values greater than 7.0 some slight increase in shrinkage temperature results, but is only slightly greater than that noted for normal collagen and is much less than that obtaining for normal collagen treated with formaldehyde. Theis and Steinhardt (10) claim that swelling and shrinkage temperature must necessarily be correlated. This is evident when the data represented by Curves C and D are studied. These particular curves show definitely that the great swelling occurring in the zone pH 5.0 to 9.5 is responsible for the decreased

TABLE I

Showing Formaldehyde-Binding Capacity of Normal and Deaminized Collagen

Normal collagen		Deaminized collagen	
Final pH	Formaldehyde fixed per gm. collagen	Final pH	Formaldehyde fixed per gm. deaminized collagen
	mM		*mM*
3.77	0.06	5.16	0.05
4.05	0.12	6.07	0.10
4.90	0.19	6.96	0.16
5.30	0.20	7.27	0.12
5.73	0.28	7.70	0.16
5.95	0.25	8.24	0.15
6.28	0.37	8.50	0.19
6.36	0.35	8.61	0.18
6.63	0.34	8.95	0.30
6.66	0.40	9.56	0.45
6.85	0.38	9.80	0.49
6.86	0.41	10.05	0.53
7.07	0.43		
7.11	0.41		
7.23	0.42		
7.48	0.41		
7.54	0.42		
7.72	0.42		
7.86	0.44		
7.94	0.44		
8.39	0.46		
8.60	0.48		
9.66	0.68		
11.36	0.93		

cohesive forces of the deaminized collagen as indicated by the shrinkage temperature in Curves G and H. These curves further show that the minimum swelling usually occurring at approximately pH 5.0 has been shifted to a more acid zone and that there is an indication of a minimum value at approximately pH 8.5 to 9.5.

The data shown by Curves E and F indicate that there is not the drastic reduction of formaldehyde fixation for deaminized collagen *versus* normal collagen as noted by Highberger and Retzsch. Curve F shows (1) a reduced aldehyde-binding capacity in the pH range 2.0 to 7.0, (2) only a slight decrease in the range pH 7.3 to 9.5, and (3) a greater reduction in binding capacity in the pH zone 10.0 to 12.0. These data are in line with the suggestions made by Theis and Lams (9, 6); namely, that lysine plays only a small rôle in the pH zone more acid than the isoionic point, and that formaldehyde binding in this region is largely confined to a reaction with the less basic imino groups of the polypeptide chains. The decrease in formaldehyde binding in this zone may be due to two causes; one, a possible change in position of the imino groups, and the other, the greater compactness of the fiber structure of the deaminized collagen. Formaldehyde binding in this acid zone is probably not in bridge-like formation since the hydrothermal stability of the collagen-formaldehyde compound is not increased, but in this case the aldehyde may be forming methylol groups with the imino groups as, $>NH + CH_2O \rightarrow >N - CH_2OH$ and not $>NHHN< + CH_2O \rightarrow >N-CH_2-N<$. Formaldehyde binding in the pH stability zone appears to be forming bonds between the polypeptide chains through reaction with the imino groups in juxtaposition since the hydrothermal stability again increases in this zone (Curve H). The aldehyde fixation shown in the pH zone 10.0 to 12.4 is believed to be that of aldehyde binding with residual amino groups of lysine, further binding with imino groups of the polypeptide chains and possibly with the guanidino groups of arginine, since arginine may have been affected by the denaturation treatment. Curve H representing shrinkage temperature also shows an increase at pH values greater than 10.6. The drastic over-all decrease in aldehyde fixation for deaminized collagen as noted by Highberger and Retzsch is not evident in the data presented in this paper.

Curves G and H representing hydrothermal stability are of real interest, since they show, by means of a physical measurement, the changes coming about through deaminization. Deaminization has shifted the broad plateau from a normal zone pH 5.5 to 9.5 to one at pH 3.0 to 6.0, and at the same time has given to the deaminized collagen-formaldehyde compound an increased hydrothermal stability, namely an increase from 58° to some 67°. This increase may well be accounted for by the increased compactness as noted from the swelling curves. Curve H representing the formaldehyde-treated deaminized collagen follows the general trend of the curves representing swelling and aldehyde fixation.

SUMMARY

Collagen denatured by deaminization has been studied with respect to its formaldehyde-binding capacity. This investigation showed: (1) formaldehyde binding is somewhat decreased in the acid and very alkaline zones, (2) the formaldehyde-binding capacity is but little decreased in the pH stability zone, (3) the hydrothermal stability is very different from that of normal collagen, and (4) swelling curves are given for normal collagen, formaldehyde-treated normal collagen, deaminized collagen, and formaldehyde-treated normal collagen.

BIBLIOGRAPHY

1. Bowes, J. H , and Pleass, W B., *J. Internat. Soc. Leather Trades Chem* , **23**, 365 (1939); **23**, 451 (1939); **23**, 499 (1939).
2. Highberger, J. H., and Retzsch, C E , *J. Am Leather Chem Assn* , **34**, 131 (1939).
3. Meunier, L , and Schweikert, E , *J. Internat Soc. Leather Trades Chem* , **19**, 350 (1935).
4. Prideaux, E. B R , and Woods, D. E., *Proc Roy. Soc. London, Series B*, **64**, 110 (1934).
5. Stendel, H., and Schumann, R., *Z physiol. Chem.*, **183**, 168 (1929).
6. Theis, E. R., *J. Biol. Chem.*, **154**, 87 (1944)
7. Theis, E. R., *J. Biol. Chem.*, **156**, 7 (1944).
8. Theis, E. R., and Jacoby, T. F , *J. Biol. Chem* , **148**, 105 (1943)
9. Theis, E. R., and Lams, M. M , *J. Biol. Chem.*, **154**, 99 (1944)
10. Theis, E. R., and Steinhardt, R G., Jr , *J. Am. Leather Chem. Assn.*, **37**, 433 (1942).
11. Thomas, A W , and Foster, S. B , *J Am. Leather Chem Assn* , **48**, 489 (1926).
12 Wiley, F. H., and Lewis, H B , *J Biol Chem* , **86**, 511 (1930)

THE COLLAGEN-QUINONE REACTION

I. FIXATION AND THERMOLABILITY AS A FUNCTION OF pH VALUES

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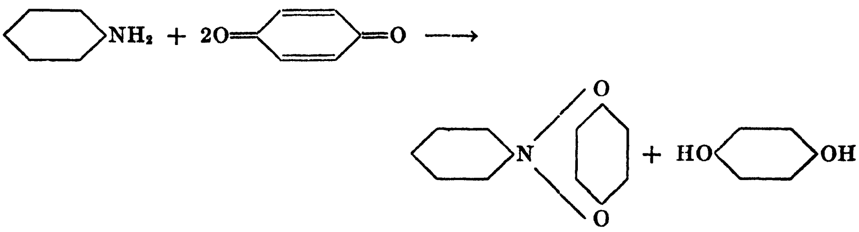
In previous papers (6, 9) we have discussed the protein-formaldehyde reaction and have postulated the reaction as being of several types: (*a*) the bridging of polypeptide chains by means of imino groups in juxtaposition; (*b*) bridging by means of amino groups in juxtaposition; and (*c*) formation of methylol groups. The reaction of a protein with formaldehyde can be very easily followed, since the particular protein-formaldehyde compound can be broken down and the fixed formaldehyde quantitatively estimated. Since quinone, like formaldehyde, contains a reactive carbonyl group and is also a substance of low molecular weight, the possibility that this substance might behave towards proteins in a manner similar to formaldehyde has been investigated.

Meunier and Seyewetz (3) first pointed out the remarkable tanning properties of *p*-benzoquinone but little study, however, was made of the reaction of quinone with proteins until 1924, when Thomas and Kelly (11, 10) carried out a series of experiments in which they determined the effect of the pH value of the solutions upon quinone fixation by collagen.

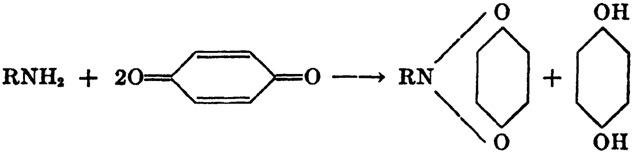
Thomas and Kelly found a sharp minimum point of quinone fixation at approximately pH 9.0. At the time they carried out this particular investigation, no accurate method of determining the pH value of quinone solutions was in use, and therefore their maximum and minimum values for quinone fixation are probably somewhat in error. Stecker and Highberger (5) in 1942 reinvestigated the work of Thomas and Kelly with, in the main, the identical technique of these investigators but determined the equilibrium pH by means of a glass electrode assembly. Stecker and Highberger, with a phosphate buffer system, found a minimum value for quinone fixation at pH 7.5. They interpreted the trend of the weight increase curves (quinone fixation) as obtained by them in the phosphate-buffered system somewhat as follows: (1) the combination of the collagen with monomeric quinone; (2) the formation of a quinone polymer which is insoluble in acid but soluble in alkali; (3) the combination of this polymer with collagen at alkaline reactions; and (4) the oxidation of the quinone or the characteristic polymer with the formation of oxidation products of high molecular weight, which combine with the collagen in acid solution.

Stecker and Highberger state that the nature of the buffer system plays an important rôle.

Meunier and Seyewetz (3) noted that the reaction between quinone and protein is accomplished by the reduction of part of the quinone to hydroquinone. They suggested that this reduction results from the oxidation of the protein by the quinone and that the oxidized protein combines with excess quinone. They pictured this reaction as similar to that of the reaction of quinone with aromatic amines.

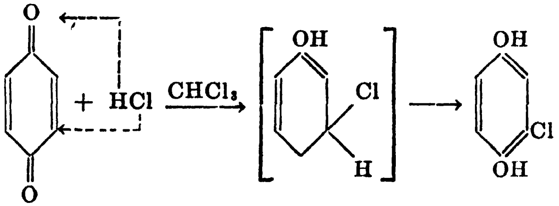


Wilson (12), applying this same reasoning, postulated the reaction between quinone and collagen as

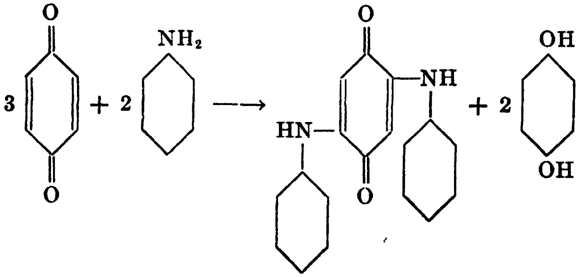


Meunier and Queriox (2) found that the ratio of hydroquinone formed to quinone destroyed was increased from that of 1:1, and this change in ratio was taken to support the contention that the quinone reaction involves the oxidation of the protein.

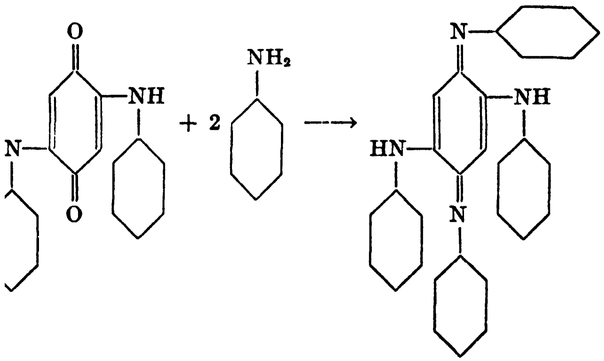
When hydrochloric acid reacts with *p*-benzoquinone in the presence of chloroform (1), the hydrogen atom of the acid adds to one of the quinonoid oxygens while the chlorine adds in the terminal position of the 1,4-system, and the unstable dihydrobenzenoid structure then enolizes to that of the fully aromatic chlorohydroquinone



When *p*-benzoquinone reacts with primary or secondary amines, hydroquinones are not formed but substituted quinones instead. This reaction is due to the relative oxidation potentials of the substituted and unsubstituted compounds, the E_0^{alcohol} for 1,4-naphthoquinone being 0.484V, while that for 2-aniline-1,4-naphthoquinone is 0.286V; thus the anilinohydronaphthoquinone formed is almost completely oxidized by the excess α-naphthoquinone and in this reaction then 2 molecules of the quinone are used in the production of 1 molecule of the substituted quinone.



The dianilinoquinone can further react with aniline with the formation of azophenin.



EXPERIMENTAL

1 gm. samples of specially prepared collagen (8) were placed in 200 ml. bottles and 100 ml. of 0.1 M phosphate buffer made 1 per cent with respect to p-benzoquinone. The series of samples varied in pH from 3.0 to 9.5. The bottles and contents were placed in a thermostat maintained at 20° for periods ranging from 24 hours to about 3 weeks. At stated intervals, the samples were agitated in order to promote equilibrium. After the time periods noted in Figs. 1 to 4 the pH at equilibrium was determined by means of a Beckman glass electrode assembly; the collagen was removed and pressed several times between blotting paper at 10,000 pounds per sq. in. It has been previously shown (6) that for all practical purposes such pressure removes the free water and any free electrolyte. We,

therefore, have assumed that all free quinone is correspondingly removed, only that which is firmly bound to the collagen itself being left behind. After being pressed, the collagen material was carefully dried in an electric oven at 105° to constant weight. The increase in weight over that of the oven-dried native collagen material was taken as the amount of quinone fixation. Another series of experiments was made under similar conditions

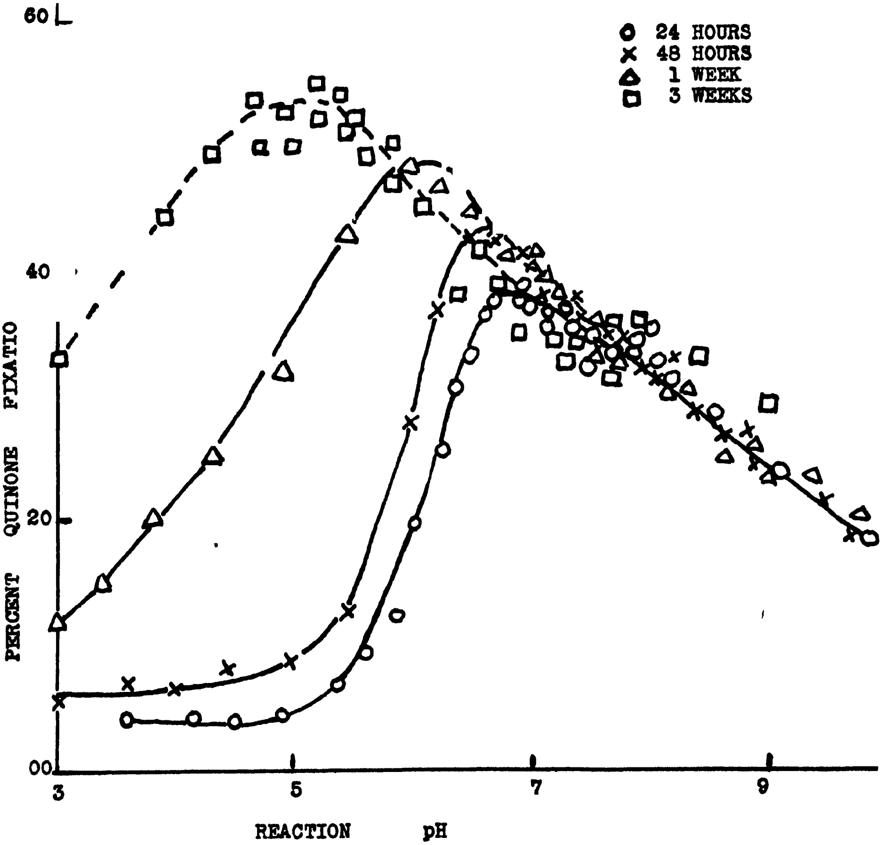


Fig 1. Showing quinone fixation by collagen, time and pH varied

but in this case collagen strips were used, since strips are necessary for the determination of shrinkage temperature. The data obtained are shown in Figs. 1 to 4.

Fig. 1 shows the quinone fixation by collagen over the pH range 3.0 to 9.5 and for periods ranging from 24 hours to 3 weeks. These data indicate the following trends: (1) there is a maximum fixation, occurring at pH 7.0

for the 24 hour curve and shifting to a pH of 5.1 for the 3 weeks curve; (2) the amount of quinone fixation at this maximum value increases with time; (3) at pH values greater than 7.0 the period of reaction makes little difference, the various curves merging with each other; and (4) no characteristic minimum point is evident at pH 7.5 as found by Stecker and Highberger.

Fig. 2 shows the data obtained over a comparatively narrow pH range but for quinone concentrations varying from 0.1 to 1.75 per cent and for a 48 hour interval. These observations suggest that the quinone concentrations play an important rôle and that the quinone fixation by collagen is a function of this concentration. The curves given in Fig. 2 show a decided shift of maximum fixation values to a more acid zone as the quinone concentration is increased.

Fig. 3 gives data relative to the shrinkage temperature of collagen treated with a 1 per cent quinone solution These data show strikingly: (1) the maximum shrinkage temperature occurs at pH 7.0 when a 1 day period of reaction obtains, and (2) with increasing periods of reaction this maximum value for shrinkage temperature changes from a sharp and striking point to one of a rather broad plateau

Fig. 4 gives data relative to the thermolability of collagen treated with quinone solutions varying in concentration from 0.1 to 1.75 per cent and for a 48 hour period of reaction. The observations indicate that at the pH of maximum shrinkage temperature, quinone concentration plays but a small rôle. However, in the more acid zone, quinone concentrations less than 0.5 per cent appear to lessen the hydrothermal stability of the collagen-quinone compound. At concentrations greater than 0.5 per cent little difference in this value is noted in the pH range 5.8 to 7.5.

DISCUSSION

Stecker and Highberger used a technique similar to that originally used by Thomas and Kelly. This technique was as follows. After the collagen-quinone reaction had reached equilibrium, the treated collagen material was washed thoroughly and until the wash water gave no further positive test for quinone with an acid-KI test solution. The washed material was then oven-dried and the increase in weight over that of the untreated collagen material was taken as fixed quinone. Wilson and Kern (13) devised this method in 1920 and used it for the estimation of "tannins" in vegetable tanning liquors. There was considerable criticism (4) of the method and no wide use of it was ever made. However, parts of the method and modifications of it can yield valuable results. It must be remembered that most reagents combine with proteins in a semireversible manner and that washing will remove part of that reagent which has fixed itself with the protein. The writer believes that Stecker and Highberger measured (1) the quinone

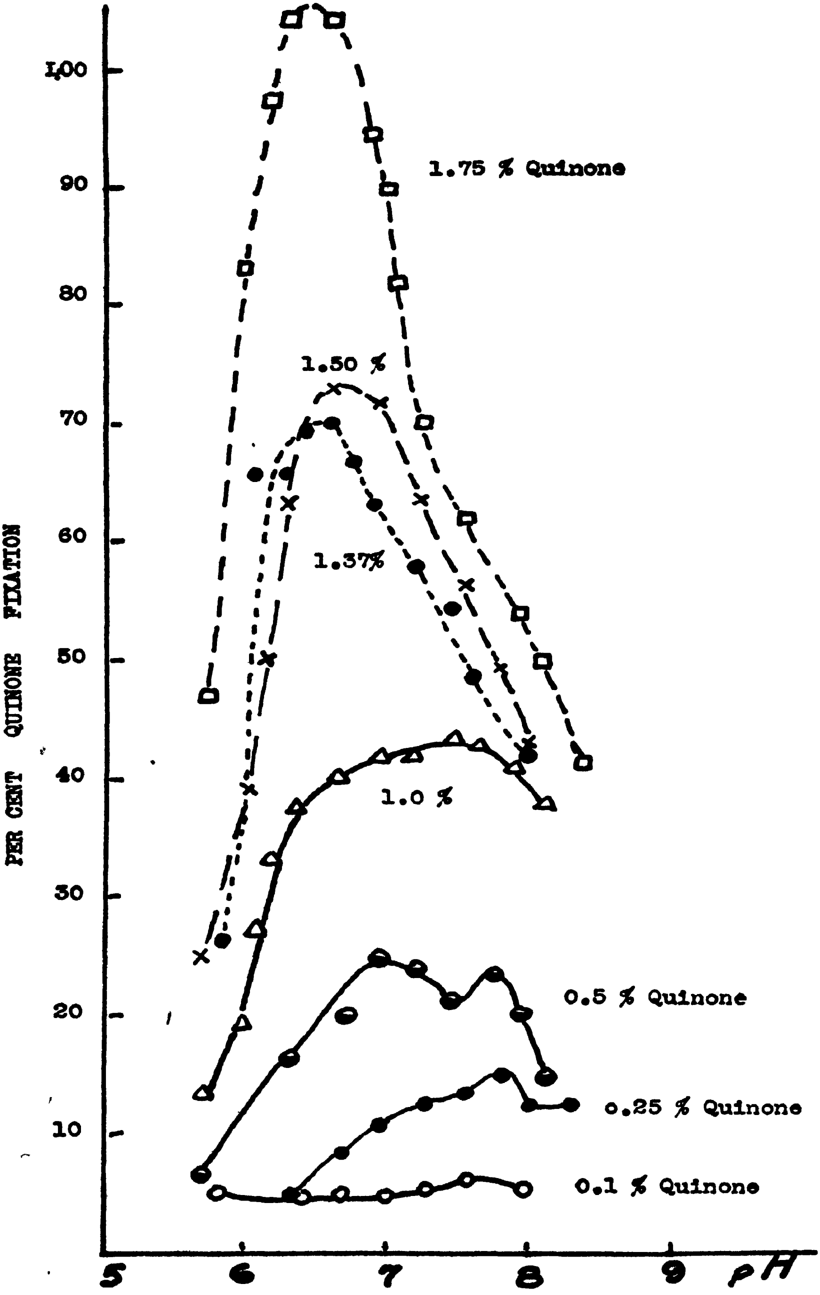


FIG. 2. Showing quinone fixation by collagen, concentration and pH varied

which was firmly and irreversibly bound to the protein and (2) the polymerized quinone which was insoluble in the wash water. In the present work, a pressing technique was employed rather than a washing, with a pressure of 10,000 pounds per sq. in., under the assumption that such pressure removed only the quinone that was in no way bound or held by the collagen. With the technique of Thomas and Kelly, Stecker and Highberger found a distinct minimum value for quinone fixation at pH 7.5 when phosphate buffer systems were used. They found no such value in borate buffer systems.

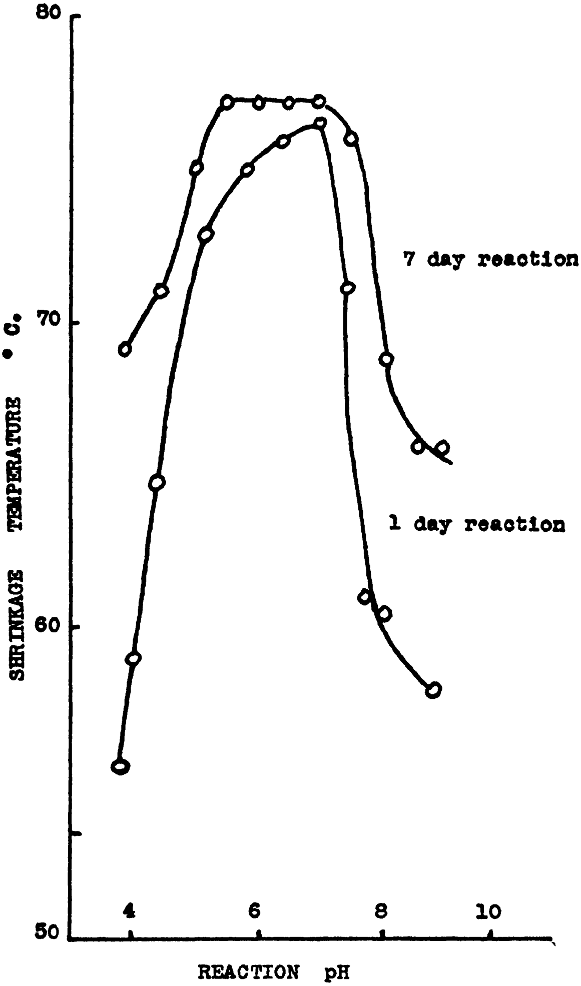


FIG. 3. Showing thermolability of quinone-treated collagen as a function of time and hydrogen ion concentration.

This difference is due, they believe, to the quinone polymer not being formed in the latter case and to the difference in oxidative processes occurring in the two systems.

The data given in the present investigation appear not to agree with that obtained by Stecker and Highberger and by Thomas and Kelly, in that no distinctive minimum value for quinone fixation was found. This appeared to be true regardless of the buffer system employed. The writer believes that the quinone monomer does not react readily with the collagen but that this monomer penetrates rapidly into the collagen, and thence through oxidation and subsequent polymerization real and positive fixation occurs. In the acid zone, oxidation and polymerization occur slowly and as a consequence quinone fixation is slow. That this postulation may be true is evident from a study of Figs. 1 and 2 These data strikingly show that in

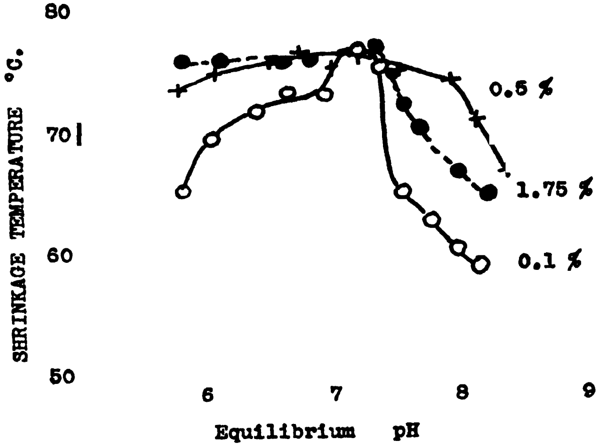
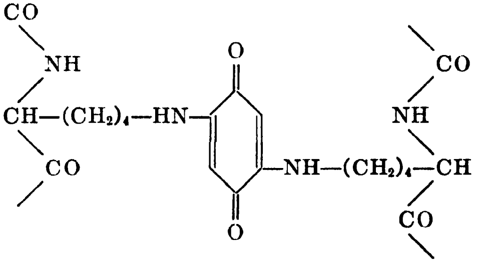


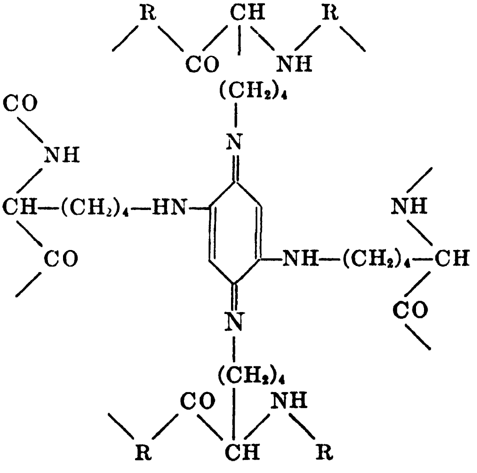
FIG 4. Showing effect of quinone concentration upon thermolability

the acid zone many days are required for real and positive quinone fixation, while at pH 7.0 fixation occurs within a very short time. In addition the value for maximum quinone fixation shifts continuously to the more acid zone with increasing time of reaction, thus strengthening the inference that oxidation and polymerization of the quinone monomer, held within the collagen, are the real cause of the high fixation values noted in this zone. At pH values greater than 7.0, oxidation and polymerization occur rapidly with the result that the larger polymeric molecules do not even penetrate the collagen material and as an end-result fixation of the quinone polymer drastically decreases. As a further substantiation, the data given in Fig. 1 show that regardless of the period of reaction quinone fixation is, to all intents and purposes, the same at pH values greater than 7.5. Borate buffer systems and pure aqueous systems show approximately the same end-result.

In another manner, Fig. 3 substantiates the conclusions outlined in the preceding paragraph. These data show that a maximum shrinkage temperature value is attained at pH 7.0 for the 24 hour reaction period, while for the 1 week period a broad plateau maximum results in the zone pH 5.5 to 7.0. These data again suggest that the longer period of contact in the more acid zone allows rapid penetration of the quinone monomer with subsequent slow oxidation, polymerization, and fixation of the polymer. Theis and Esterly (7) have defined the "shrink temperature" as the point at which the increasing disruptive tendencies exceed the diminishing cohesive forces; and thus the "shrinkage temperature" is actually a measure of the structural strength of the collagen expressed in arbitrary units. Collagen in the moist state shows a shrinkage temperature of approximately 58° over the pH range 5.0 to 8.5. Collagen treated with quinone solutions has a shrinkage temperature ranging from 58° at pH 4.0 to 76.5° at pH 7.0, and thus it may be stated that quinone fixation with collagen definitely increases the structural strength of the collagen.



(I)



(II)

In the early part of this paper, reactions of quinone with aromatic amines were discussed. If this same line of reasoning is applied to the reaction of *p*-benzoquinone with collagen, Formulas I and II result. Thus the increased structural strength of the collagen after combination with quinone might be stated to be due to the bonding between polypeptide chains by means of the reaction of the reactive centers of quinone with amino groups in juxtaposition in some such manner as pictured above. That both reactions take place can be proved by treating the freshly prepared collagen-quinone compound with a solution of acid potassium iodide, by which treatment free odine is formed. The aged collagen-quinone compound gives only a trace of iodine upon such treatment.

Fig. 2 would appear to indicate that the quinone-collagen reaction is of two types; the one very rapid and attaining maximum fixation at approximately pH 7.0, and the other a slow oxidation and polymerization with subsequent increased fixation and structural stability over a much wider pH zone. A close study of Figs. 3 and 4 will demonstrate more fully the two types of reaction taking place. A comparison of data given in Figs. 2 and 4 shows in a very striking manner that the amount of quinone fixed plays only a small rôle in giving to the collagen increased structural stability. A 0.1 per cent quinone solution at pH 7.3 shows some 6.0 per cent quinone fixation, but gives to the collagen a shrinkage temperature of 77° with a 48 hour reaction period. On the other hand a 1.0 per cent quinone solution under like conditions gives some 42 per cent quinone fixation, but the shrinkage temperature remains essentially the same.

Undoubtedly the pH zone in and around 7.0 is a critical one for collagen. In this zone zero acid and base fixation occur (8). It is also in this zone that the second point of minimum swelling obtains. It would therefore be reasonable to expect that quinone fixation in this zone would be most easily reversed by washing. It would seem probable that the results obtained by Thomas and Kelly and by Stecker and Highberger do not represent the total fixation of quinone by collagen but only that of the quinone firmly and irreversibly bound.

SUMMARY

The collagen-quinone reaction has been investigated relative to the effect of pH upon quinone fixation and thermolability. The investigations herein described show the following: (1) the reaction is a 2-fold one, in that the initial reaction is rapid while the second requires time for oxidation, polymerization, and fixation; (2) the amount of quinone fixation is no criterion of structural stability; (3) quinone fixation is a function of the concentration and the reaction pH; (4) maximum quinone fixation is a function of the pH and of the period of reaction; (5) oxidation and polymerization of

the quinone within the collagen are of real importance in the estimation of fixation in the acid zone; (6) a possible reaction mechanism is suggested; and (7) greater fixation of quinone than noted by Stecker and Highberger is indicated and is undoubtedly due to the difference in technique employed.

BIBLIOGRAPHY

1. Fieser, L. F., and Fieser, M., Organic chemistry, Boston (1944).
2. Meunier, L., and Queriox, M., *J. Internat. Soc. Leather Trades Chem.*, **9**, 26 (1925).
3. Meunier, L., and Seyewetz, A., *Compt. rend. Acad.*, **146**, 987 (1908); *Collegium*, **346**, 59 (1909); **375**, 319 (1909).
4. Schultz, G. W., *J. Am. Leather Chem. Assn.*, **15**, 451 (1920).
5. Stecker, H. C., and Highberger, J. H., *J. Am. Leather Chem. Assn.*, **36**, 226 (1942).
6. Theis, E. R., *J. Biol. Chem.*, **154**, 87 (1944).
7. Theis, E. R., and Esterly, A. R., *J. Am. Leather Chem. Assn.*, **35**, 563 (1940).
8. Theis, E. R., and Jacoby, T F., *J. Biol Chem* , **146**, 163 (1942).
9. Theis, E. R., and Lams, M. M., *J. Biol. Chem.*, **154**, 99 (1944).
10. Thomas, A. W., and Foster, S. B., *J. Am. Chem. Soc.*, **48**, 489 (1926).
11. Thomas, A. W., and Kelly, M. W., *Ind. and Eng. Chem.*, **16**, 925 (1924); **18**, 383 (1926).
12. Wilson, J. A., The chemistry of leather manufacture, New York, 2nd edition, **2** (1929).
13. Wilson, J. A., and Kern, E. J., *Ind. and Eng Chem.*, **13**, 772 (1921); **12**, 465 (1920)

FATTY ACID METABOLISM

II. THE BREAKDOWN OF CARBOXYL-LABELED BUTYRIC ACID BY LIVER TISSUE

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In a previous communication (1) it was shown that the presence of excess C^{13} in acetoacetic acid, formed from the breakdown of *n*-octanoic acid containing isotopic carbon in the carboxyl group, was inconsistent with the classical β oxidation theory. The observed equal distribution of the excess C^{13} between the carboxyl and carbonyl carbon atoms also precluded the possibility of multiple alternate oxidation. The results indicated that a breakdown to a 2-carbon unit occurs, with subsequent random condensation to the ketone bodies, as shown in the following equation.

$$(1) \qquad C_7H_{15}C^{13}OOH \rightarrow 4CH_3C^{13}OOH \rightarrow 2CH_3C^{13}OCH_2C^{13}OOH$$

However, the data did not rule out an alternate mechanism; namely, that disruption of the fatty acid chain into 2-carbon units may proceed only to the butyric acid stage, the latter being oxidized to acetoacetic acid without fission of a carbon-to-carbon linkage (Equation 2).

$$(2) \qquad \begin{array}{cccc} C_7H_{15}C^{13}OOH \longrightarrow & CH_3CH_2CH_2COOH & + & 2CH_3C^{13}OOH \\ & \downarrow & & \downarrow \\ & CH_3COCH_2COOH & & CH_3C^{13}OCH_2C^{13}OOH \end{array}$$

To determine to what extent butyrate is transformed to acetoacetate by fission and recombination, and to what extent by direct β oxidation, experiments were performed in which *n*-butyric acid labeled with C^{13} in the carboxyl group was employed as a substrate.

EXPERIMENTAL

Preparation of Carboxyl-Labeled n-Butyric Acid—The carboxyl-labeled *n*-butyric acid was prepared by the same method employed for the preparation of carboxyl-labeled *n*-octanoic acid, described previously (1). It contained 2.10 per cent C^{13}, corresponding to an excess C^{13} content of 4.24 per cent in the carboxyl carbon.

Incubation with Liver Slices—The techniques employed were essentially the same as those described in the previous paper (1), except that the capacity of the system was doubled by performing the reaction in two 125 ml. Warburg type flasks connected by a T-tube to a single gas burette. Each flask contained 1.25 gm. of liver slices immersed in 30 ml. of approximately

0.01 M solution of the fatty acid in the medium previously described (1). At the end of the 2 hour incubation period, the alkali-soaked filter papers were removed and the contents of the flask combined and centrifuged. The vessels and tissues were rinsed with dilute NaOH solution, followed by two successive rinsings with small amounts of water. The supernatant liquid and washings were pooled.

The diluted medium was divided into two portions. Half was used for isolation of the acetone-mercury complexes and for the collection of CO_2 evolved during the decomposition of the ketone bodies as previously described (1). After removal of the ketone bodies, the volatile substances were distilled with steam, and an aliquot portion of the residue was oxidized by the procedure of Osburn and Werkman (2) (carried out only in Experiment 2). The CO_2 obtained was analyzed for C^{13}. The second half of the medium was steam-distilled directly and the Duclaux constants of the volatile acid determined according to the procedure of Friedemann (3). In Experiment 2, the neutral, volatile substances were separated from the volatile acid by distillation of the neutralized solution. The acids (as the sodium salts) were oxidized and the C^{13} content of the CO_2 determined. The acetone contained in the volatile fraction was precipitated with mercuric sulfate and oxidized to CO_2. The filtrate, representing the volatile non-acetone fraction, was oxidized (4) and the CO_2 analyzed for its C^{13} content.

TABLE I

*Distribution of C^{13} in Products of Incubation of Carboxyl-Labeled Butyric Acid with Liver Slices**

Time, 2 hours; temperature, 37.5°; O_2; pH, 7.3.

	Experiment 1		Experiment 2	
	mM	*C^{13} per cent*	*mM*	*C^{13} per cent*
Acid utilized	0.192	2.10	0.155	2.10
Acetoacetic acid, acetone	0.093	1.42	0.0876	1.33
" " carboxyl	0.103	2.83	0.1213	2.92
Hydroxybutyric acid, acetone	0.063	1.24	0.0419	1.27
" " carboxyl		1.32	0.290	1.15
Respiratory CO_2	0.239	1.50	0.197	1.45
Neutral volatile substances other than acetone			0.0504	1.37
Non-volatile material			0.710	1.15
Fat-free tissue		1.07	404 mg.	1.04
Tissue lipids			130 "	1.05

* The C^{13} content is expressed as atoms per cent C^{13}, from the equation, per cent C^{13} = (moles C^{13}/moles C^{12} + moles C^{13}) × 100.

A portion of the liver slices was extracted with alcohol and ether and C^{13} analyses carried out on aliquots of the lipid extract and the fat-free tissue. The remaining slices were macerated and steam-distilled for recovery of any acid which may have been adsorbed. None was found, however.

Results

The results of two experiments, in which carboxyl-labeled *n*-butyric acid was incubated for 2 hours with liver slices from rats fasted for 24 hours, are recorded in Table I. Of the 0.376 and 0.479 mM of butyric acid employed in the respective experiments, 0.189 and 0.324 mM were recovered, corresponding to a utilization of 51 and 32 per cent of the original substrate.

Respiratory CO_2—The respiratory CO_2 contained 1.50 and 1.45 per cent C^{13}, representing excesses of 0.46 and 0.41 per cent respectively. Since the butyric acid contained an excess of $(2.10 - 1.04)$, or 1.06 per cent, the fraction of the respiratory CO_2 derived by complete oxidation of the substrate was $0.46 \times 100/1.06$ and $0.41 \times 100/1.06$ respectively, or 42 and 39 per cent of the total respiratory CO_2.

Acetoacetic Acid—In Experiment 1, the acetone derived by breakdown of the acetoacetic acid contained 1.42 per cent C^{13}, representing an excess of $0.38 \times 3 = 1.14$ per cent in the carbonyl carbon. The CO_2 derived from the carboxyl carbon contained 2.83 per cent C^{13}, an excess of 1.79 per cent. In Experiment 2 the excess C^{13} contents of the carbonyl and carboxyl carbons were 0.87 and 1.88 per cent respectively.

As a check on the specificity of the acetone precipitation procedure, in Experiment 2 a portion of the acetone was freed from non-volatile substances and volatile acids as described in the experimental part. The mercury complex obtained from this neutral volatile fraction was the same in quantity and C^{13} content (1.31 per cent) as that obtained by the direct precipitation procedure.

β-Hydroxybutyric Acid—The acetone obtained by oxidation of the β-hydroxybutyrate in Experiment 1 contained 1.24 per cent C^{13}, representing an excess C^{13} content of $0.20 \times 3 = 0.60$ per cent in the β-carbon atom. In Experiment 2 the corresponding value was $0.23 \times 3 = 0.69$ per cent.

The values recorded in Table I for the C^{13} content of the CO_2 obtained by oxidation of hydroxybutyrate do not represent the true values for the carboxyl carbon of hydroxybutyrate, since the CO_2 from this source was diluted by CO_2 from other substances present in the medium. This is clearly shown in Experiment 2, in which oxidation of hydroxybutyrate yielded 0.042 mM of acetone as compared with 0.290 mM of CO_2. However, the absolute quantity of C^{13} in this CO_2 fraction, $0.290 \times (1.15 - 1.04)/100 = 3.2 \times 10^{-4}$ mM, is approximately equal to the absolute amount of C^{13} in the acetone fraction, $0.042 \times 3\ (1.27 - 1.04)/100 = 2.9 \times 10^{-4}$ mM. This indicates that the medium contained no appreciable amounts of easily oxidizable substances containing excess C^{13} other than hydroxybutyric acid.

Recovery of C^{13}—The percentage of C^{13} represented by the butyric acid utilized, appearing in the various fractions, is given in Table II. In Experiment 1 only 60.9 per cent of the total was accounted for as ketone bodies and respiratory CO_2. In Experiment 2, therefore, we carried out C^{13} determinations on the carbon contained in the non-volatile and volatile neutral fractions, obtained as described in the experimental part. These fractions accounted for 13.8 and 5.8 per cent, respectively, of the total utilized C^{13}. Their nature is as yet unknown. The total C^{13} accounted for in this experiment was 87.0 per cent. The tissue constituents contained no perceptible excess of C^{13}, a result to be expected, considering the small quantities of excess C^{13} which may have been present in the relatively large mass of tissue carbon.

TABLE II

Percentage of C^{13} Utilized As Butyric Acid Appearing in Fractions Isolated from Incubation of Carboxyl-Labeled Butyric Acid with Liver Slices

	Experiment 1	Experiment 2
Acetoacetic acid	35.7	45.8
Hydroxybutyric acid	11.7	9.3
Respiratory CO_2	13.5	12.3
Neutral volatile substances other than acetone	*	5.8
Non-volatile material	*	13.8
Tissue constituents	0	0
Total accounted for	60 9	87 0

* Not determined.

DISCUSSION

Mechanism of Ketone Body Formation from Butyric Acid—Two mechanisms may be considered for the formation of ketone bodies from butyric acid: direct oxidation of the β-carbon atom without disruption of the molecules, and β oxidation to 2-carbon intermediates, followed by condensation to ketone bodies. With carboxyl-labeled butyric acid, the two reactions lead to different distributions of the excess isotope in the acetoacetic acid, as indicated in Equations 3 and 4.

$$CH_3CH_2CH_2C^{13}OOH \rightarrow CH_3COCH_2C^{13}OOH \tag{3}$$

$$CH_3CH_2CH_2C^{13}OOH \rightarrow 2CH_3C^{13}OOH \rightarrow CH_3C^{13}OCH_2C^{13}OOH \tag{4}$$

In Experiment 1 the carbonyl carbon had an excess C^{13} content of 1.14 atom per cent and the carboxyl carbon 1.79 atom per cent, a total of 2.93 moles per cent. Before the quantitative significance of these values is discussed, several reasonable assumptions must be made. The first is that the difference between the C^{13} content of the original butyric acid and the acetoacetic acid is a measure of the dilution of acetoacetate arising from the substrate by non-isotopic acetoacetate from the tissue constituents. For example, in Experiment 1 the acetoacetate contained 2.93 moles per cent C^{13} excess, whereas the substrate contained 4.24 moles per cent excess. Hence, $2.93 \times 100/4.24 = 69$ per cent of the acetoacetate was derived from the substrate and 31 per cent came from the tissue. Similarly derived, the corresponding values in Experiment 2 were 65 and 35 per cent. The second assumption is that, regardless of the mechanisms by which butyric acid is transformed to acetoacetic acid, the total excess C^{13} content of the latter would be the same. This follows from the fact that the reaction does not involve a net gain or loss of carbon. Thus, if the reaction proceeded by direct oxidation without fission, the acetoacetate would contain 2.93 moles per cent C^{13} excess, exclusively in the carboxyl carbon. If the reaction proceeded entirely by fission and coupling, the acetoacetate would again contain 2.93 moles per cent excess C^{13}, but it would be divided equally between the carbonyl and carboxyl carbons, each containing 1.465 atom per cent C^{13} excess.

It follows, therefore, that in the formation of acetoacetate from butyrate the percentage arising by fission and coupling would be

$$\frac{2 \times \text{carbonyl } C^{13} \text{ excess} \times 100}{\text{Total acetoacetate } C^{13} \text{ excess}} = \frac{2 \times 1.14 \times 100}{2.93} = 78\%$$

Similarly, the percentage of the acetoacetate arising from butyrate by direct oxidation would be

$$\frac{(\text{Total acetoacetate } C^{13} \text{ excess} - 2 \times \text{carbonyl } C^{13} \text{ excess}) \times 100}{\text{Total acetoacetate } C^{13} \text{ excess}} = \frac{2.93 - (2 \times 1.14) \times 100}{2\ 93} = 22\%$$

The corresponding values, similarly derived, for Experiment 2 are 64 per cent by fission and coupling and 36 per cent by direct oxidation.

The fact that acetoacetic acid formed in these experiments arises partly by direct oxidation of the β-carbon atom, and partly by breakdown into smaller fragments followed by recombination, does not necessarily signify two separate oxidation pathways for butyric acid. It is possible that the first step is the formation of acetoacetic acid, which may be in equilibrium with the 2-carbon intermediary, as indicated in Equation 5.

$$CH_3CH_2CH_2COOH \rightarrow CH_3COCH_2COOH \rightleftarrows 2CH_3COOH \tag{5}$$

This concept is supported by Lehninger's (5) finding of an enzymatic breakdown of acetoacetic to acetic acid in muscle and bacteria. If such an interconversion occurs, it cannot be very rapid; otherwise both types of acetoacetic acid could not have been demonstrated.

Comparison of Acid Before and After Incubation—The Duclaux constants of the volatile fatty acid fraction after incubation with the liver slices were identical with those of the original carboxyl-labeled butyric acid; hence small amounts, if any, of other volatile acids were present. The excess C^{13} contents of the original substrate and of the volatile acid isolated after incubation were also identical, the respective values being 1.06 and 1.07 atom per cent excess, from which we can conclude that the substrate was not diluted by butyric acid arising by oxidation of tissue fatty acids. However, evidence that such oxidation occurred during these experiments is deduced from the following considerations. In Experiment 1, the excess C^{13} content of the acetoacetic acid was $(1.14 + 1.79) = 2.93$ moles per cent, and in Experiment 2 $(0.87 + 1.88) = 2.75$ moles per cent, whereas the original butyric acid had an excess C^{13} content of 4.24 moles per cent. Therefore, of the total acetoacetic acid, $2.93 \times 100/4.24 = 69$ per cent and $2.75 \times 100/4.24 = 65$ per cent, respectively, were derived from the substrate, and 31 and 35 per cent by oxidation of tissue fatty acids. Clearly, butyric acid either is not an intermediate in the breakdown of these tissue constituents, or, if formed, is further metabolized without mixing with the isotopic butyric acid employed as substrate.

Other Possible Oxidation Pathways of Butyric Acid—Of the total respiratory CO_2 with a C^{13} content of 1.50 and 1.45 per cent, respectively, the amount derived by oxidation of butyric acid was $(1.50 - 1.04) \times 0.239/1.06 = 0.104$ mM in Experiment 1, and $(1.45 - 1.04) \times 0.199/1.06 = 0.076$ mM in Experiment 2. This CO_2 may arise by subsequent breakdown of acetoacetic acid or by some other oxidative pathway of butyrate as yet undetermined. Cohen and Stark (6) observed, in liver slices from fasted rats, a breakdown rate for acetoacetate of 0.80 microliter per mg. of dry tissue per hour. If the wet weight is assumed to be 5 times that of the dry weight, this value is equivalent to $(0.80 \times 2500 \times 2)/(5 \times 22{,}400) = 0.036$ mM per 2.5 gm. of wet tissue per 2 hours. If we assume further that all of the utilized acetoacetate was converted to CO_2, $0.036 \times 4 = 0.144$ mM of CO_2 would be expected. Since this figure is larger than the observed values of 0.104 and 0.076 mM of CO_2 derived from *n*-butyric acid in the respective experiments, it is clear that all of the excess C^{13} appearing in the respiratory CO_2 may be accounted for by breakdown of ketone bodies.

That butyric acid may be metabolized, to a small degree, by some other reaction is indicated by the presence of 13.8 per cent of the utilized C^{13} in the residual, non-volatile, carbon-containing material remaining after removal of the ketone bodies and volatile acids. The composition of this fraction is being studied. A small portion (5.8 per cent) of the utilized C^{13} was found in the volatile neutral fraction after the acetone had been removed. But in view of the small quantities of carbon involved (0.05 mM), associated with a large error in the C^{13} determination, the significance of this finding is questionable.

SUMMARY

A study of the breakdown of carboxyl-labeled butyric acid by liver tissue indicates that this substance is converted to ketone bodies mainly by fission into 2-carbon chains with subsequent recombination, and to a lesser extent by direct β oxidation.

The butyric acid isolated at the close of the experiment was not diluted by butyric acid from other sources.

All of the excess C^{13} of the respiratory CO_2 can be accounted for as the result of the breakdown of ketone bodies, and does not necessarily represent a different oxidation pathway in the breakdown of butyric acid.

We wish to express our thanks to Dr. H. C. Urey of Columbia University and to the Houdry Laboratories of the Catalytic Development Corporation for their cooperation in this work.

BIBLIOGRAPHY

1. Weinhouse, S., Medes, G., and Floyd, N. F., *J. Biol. Chem.*, **155**, 143 (1944).
2. Osburn, O. L., and Werkman, C. H., *Ind. and Eng. Chem., Anal. Ed.*, **4**, 421 (1931–32).
3. Friedemann, T. E., *J. Biol. Chem.*, **123**, 161 (1938).
4. Van Slyke, D. D., *J. Biol. Chem.*, **32**, 455 (1917).
5. Lehninger, A. L., *J. Biol. Chem.*, **143**, 147 (1942).
6. Cohen, P. P., and Stark, I. E., *J. Biol. Chem.*, **126**, 97 (1938).

ISOLATION OF LYSOZYME FROM EGG WHITE

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In 1922 Fleming (1) applied the term lysozyme to a bacteriolytic agent found in the tissues of a number of species of animals. The highest concentration of the agent has been found in hen's egg white which, in a dilution of about 1:500,000, has the power to dissolve a thick suspension of test organism.

Previous chemical work has been concerned only with the active principle from egg white. Available evidence, contributed mainly by Abraham (2), indicates that lysozyme is a basic protein with a molecular weight near 18,000. Abraham reported that it is stable when heated in acid solution but very heat-labile in alkali. Acetone, ether, and alcohol precipitate the active material from aqueous solution without destroying it. This latter property is the basis of the methods of preparation devised by Roberts (3) and Meyer *et al.* (4). In these methods the initial purification is obtained by rendering most of the egg white proteins insoluble by selective acetone denaturation at the alkaline reaction of native egg white. In the method of Meyer *et al.* (4) the precipitated proteins are first extracted with a mixture of alcohol and aqueous acetic acid This step is followed by concentration of the extract under a vacuum, precipitation with alcohol, solution of the precipitate, and isoelectric precipitation of inactive protein with sulfuric acid The lysozyme is then precipitated by flavianic acid. Repeated extraction of the dye-lysozyme complex with alcoholic ammonia liberates the active portion of the complex. The insoluble residue containing lysozyme is freed from ammonia by washing with alcohol and ether, followed by drying under a vacuum

Investigations dealing with the therapeutic usefulness of lysozyme have been reported by Russian workers (5). They report favorable results from treatment of ulcers of the eye, postoperative infections, burns, infected wounds, and sinus infections. It appears, therefore, that lysozyme might find wide application if sufficient material could be made available in purified form. The present communication reports the results of work which was initiated for the purpose of developing a method for the preparation of large quantities of lysozyme. The method reported here makes possible the recovery of lysozyme from egg white in high yield, and the product appears to be essentially pure. In addition, the product has been obtained in apparently pure crystalline form.

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EXPERIMENTAL

Purification of Lysozyme by Salt Fractionation—Attempts were made to purify lysozyme by fractionating the egg white with ammonium sulfate, but with indifferent success. The characteristic of lysozyme that militated against salt fractionation was its adsorption in varying degrees on inactive protein precipitates. The effect of pH on the ammonium sulfate precipitation was investigated but failed to reveal conditions that would prevent adsorption of lysozyme. Likewise, dialysis against buffers of different strength and hydrogen ion concentration did not prevent adsorption. By repeated solution and reprecipitation of euglobulin fractions containing adsorbed lysozyme, preparations with an activity 25 to 30 times that of egg white, on the dry weight basis, were obtained in low yield.[1]

Purification of Lysozyme by Adsorption and Elution Methods—Fleming (1) reported that lysozyme is readily adsorbed on charcoal, cellulose, and porcelain, and subsequent studies have shown that lysozyme is adsorbed tenaciously on many substances. Wolff (7) found that norit, kaolin, silicic acid, and filter paper adsorb the active material. He was unable, however, to effect elution from kaolin or charcoal, irrespective of pH. In studies on the purification of lysozyme, the Russian investigators, Buyanovskaya and Jermol'eva (5), attempted to make use of the ready adsorbability of lysozyme on clay. At pH values ranging from 5.5 to 9.0, lysozyme was adsorbed on kaolin suspended in buffer solutions, including phosphates, glycocholates, acetates, and dilute ammonia solutions. Treatment of the kaolin-lysozyme complex with various buffer solutions ranging from pH 4.5 to 14 did not elute the active material. Attempts to separate the positively charged lysozyme from the negatively charged clay particles by cataphoresis were unsuccessful.

We investigated the capacity of several adsorbing materials to bind lysozyme. Among these were bentonite (a very finely divided montmorillonite clay), Supersorb (a synthetic zeolite), Panther Creek bentonite, and Duolite (a synthetic resinous cation exchanger). Of the substances investigated, bentonite was found to be the most efficient adsorbent for lysozyme from native egg white. 1 gm. of the material removed the active substance from 80 to 100 cc. of egg white. Table I gives the data from a number of representative experiments.

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[1] While this preparation was not investigated further, comparison of its activity with that of crystalline lysozyme obtained later showed that it was of high purity. The method of Boasson (6) was used for assay purposes. By this method the rate of lysis of a suspension of killed *Micrococcus lysodeikticus* organisms is measured as indicated by the rate of change of light transmission recorded by a photoelectric colorimeter. Under standardized conditions a given change in galvanometer reading over a certain time interval corresponds to a definite amount of lytic agent. A lysozyme unit is defined as the amount of lysozyme in 1 ml. of a standard sample of liquid egg white. However, the amount of lysozyme in fresh egg white has been found to be very constant; so the unit might be stated to be the amount present in 1 ml. of fresh egg white. As routinely carried out in our work the method was reliable to approximately 15 per cent.

We have confirmed the results of previous workers in that we were unable to elute the lysozyme with aqueous inorganic buffer solutions. Potassium chloride solution and 6 M urea solution likewise did not remove any active material. Since organic bases are known to be strongly adsorbed by montmorillonite clays, solutions of *o*-phenylenediamine and *p*-phenylenediamine were tried but the amines were adsorbed on the clay without eluting lysozyme. However, a mixture of 20 per cent pyridine in 2 per cent aqueous acetic acid did elute about 30 per cent of the adsorbed activity. The pH of this solution, as determined by a glass electrode, was 6.0.

TABLE I

Adsorption of Lysozyme by Bentonite

Volume of egg white	Lysozyme	Bentonite per liter egg white	Lysozyme adsorbed	
ml.	*total units*	*gm*	*units*	*per cent*
500	500	10	480	96
2000	1850	10	1600	87
1200	1200	10	1190	99
2000	2000	15	1980	99
2000	2000	12.5	1700	85
3000	3000	15	2900	97
2000	1900	15	1850	97
2000	2000	15	1950	97

Investigation of the effect of pH on the elution of lysozyme by pyridine solutions proved that it could be eluted very efficiently at the proper pH. In these experiments acetic acid was replaced by sulfuric acid in order to extend the pH range. Aliquots of a suspension of clay on which lysozyme had been adsorbed were stirred with pyridine-sulfuric acid solutions at various pH values. The eluates were removed from the clay and assayed for lytic activity.

The elution of lysozyme was found to be specific with regard to the pH of the eluting solution (Fig. 1). When the pH is lowered, removal of the active material begins at pH 6.2, rises sharply to a maximum at pH 5.0, and falls off to zero at pH 3.0. Appreciable amounts of inactive protein are eluted by pyridine or pyridine-sulfuric acid solutions at pH values above 6.2, thereby effecting a marked purification of the adsorbed bactericide.

Based on the facts reported above, the following method was devised for separating lysozyme from other egg white proteins. To each liter of egg white are added 150 ml. of a 10 per cent suspension of bentonite in 1 per cent KCl and the mixture is stirred vigorously (foaming being avoided) for 3 to 5 minutes. The clay is separated from the suspension in a Sharples centrifuge and washed with 0.5 M phosphate buffer at pH 7.5 to remove egg white mechanically held in the clay mass. The clay is next washed three times with 300 ml. (total 900 ml.) of a 5 per cent aqueous solution of pyridine to remove inactive adsorbed proteins. Elution of lysozyme is then accomplished by washing the clay twice, each time with 300 ml. of a 5 per cent aqueous solution of pyridine, which has been adjusted to pH 5.0 (glass electrode) by the addition of sulfuric acid. Removal of the eluates from the clay is accomplished by centrifuging in a batch type centrifuge. For best results elution should follow adsorption within 24 hours.

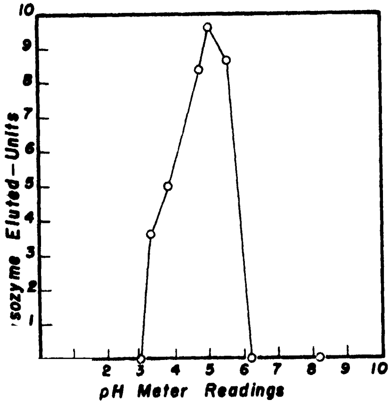

FIG 1 Effect of pH on elution of lysozyme from bentonite

Typical data on distribution of protein solids, yield of lysozyme, and activity of the preparations obtained are given in Table II. These data show that as high as 90 per cent of the lysozyme present in egg white can be obtained by this process in a concentration of 35 to 40 times that of the original egg white solids. The activity of the material is constant from one preparation to another within the limits of error of the assay method.

Concentration of the active material contained in the eluates at pH 5.0 may be accomplished in a number of ways. Addition of ammonium sulfate to a concentration of 2.6 M precipitates the active substance, which may be collected by centrifugation or filtration. The precipitate readily dissolves in distilled water and the solution may be freed from salt by dialysis. Drying the solution in the frozen state gives a white solid, of the activity indicated, which remains stable at room temperatures for several months. Concentration can be effected also by precipitation with 75 per cent alcohol or acetone, preferably in the cold, followed by dialysis.

We have found it more convenient to prepare the dry lysozyme powder in the following manner. The active eluates are placed in cellophane bags and dialyzed against running tap water until no odor of pyridine remains. The dialysis is then completed against running distilled water for 24 hours. The solute, after being dried from the frozen state, is a light, fluffy, stable powder.

Fractionation of Purified Lysozyme with Ammonium Sulfate—In an attempt to purify the active material further, two 1 per cent solutions of the eluted lysozyme in distilled water, adjusted to pH 5 and 7, were fractionated with ammonium sulfate. The salt was added by dialysis through a rotating membrane (8) at 1°, and precipitates were removed at increments of 0.2 M ammonium sulfate concentration and the solids and activity determined on the precipitates and filtrates (Table III). No significant concentration of activity was observed in any of the fractions, the activity of each being essentially that of the starting-material within the limits of the determinations. The least and the most soluble fractions appear to have less activity than the rest of the material but the amounts of solids in these fractions were so small that the accuracy of the analysis is questionable. It appears from these experiments that the preparations obtained on elution were grossly homogeneous.

TABLE II

*Elution of Lysozyme from Bentonite Clay**

Eluant	Experiment No.	Eluted protein solids per unit adsorbed lysozyme	Elution	Units per gm. protein solids
		gm	*per cent*	
Phosphate buffer eluates, pH 7-8	1	0.0046	<2	10
	2	0.0055		
	3	0.0047		
	4	0.0033		
Alkaline pyridine eluates, pH 7-8	1	0.0014	<2	10
	2	0.0018		
	3	0.0020		
	4	0.0017		
Pyridine-sulfuric acid eluates, pH 5	1	0.0033	93	285
	2	0.0027	93	320
	3	0.0027	89	330
	4	0.0032	95	300

* Egg white contains 0.1 to 0.12 gm. of protein solids per ml. and 8 to 10 lysozyme units per gm. of protein.

Action of Enzymes on Lysozyme Preparation—It has been reported that lysozyme is not destroyed by proteolytic enzymes (5). Since it is non-dialyzable, it should be possible to remove digestible contaminating proteins by enzyme degradation followed by dialysis. Accordingly, 10 ml. portions of a lysozyme solution containing 8 units per ml. were digested for 24 hours at 30° with bacterial proteinase (pH 7.4), mold proteinase (pH 7.4), trypsin (pH 7.4), papain activated with cysteine (pH 6.0), and pepsin (pH 2.0). After the activity of each digest had been determined, each was dialyzed against distilled water for 16 hours and the solids determined. The activity per unit of undigestible material was then calculated.[2]

No significant loss of activity took place on digestion with any of the enzymes, with the exception of pepsin. Similarly no marked loss of material took place on dialysis after digestion except in the case of the pepsin-treated material. The calculated activity of the undigested material was in all cases not significantly different from the determined activity before digestion (Table IV).

TABLE III

Ammonium Sulfate Fractionation of Lysozyme at pH 5 and 7

$(NH_4)_2SO_4$ concentration	Fraction	Assay, units		Protein solids, gm		Units per gm protein	
		pH 5	pH 7	pH 5	pH 7	pH 5	pH 7
moles per l							
	Starting material	1090		3.43		318	
1.4	Ppt.	45	10	0.18	0.05	250	200
1.4	Filtrate	980	930	3.18	3.31	308	281
1.6	Ppt.	316	223	1.24	0.81	255	275
1.6	Filtrate	505	586	1.63	2.00	310	293
1.8	Ppt.	245	305	0.78	1.04	314	293
1.8	Filtrate	246	358	0.93	1.06	265	338
2.0	Ppt.	125	160	0.41	0.53	305	304
2.0	Filtrate	141	147	0.40	0.48	352	306
2.4	Ppt.	83	122	0.28	0.39	296	311
2.4	Filtrate	20	20	0.20	0 18	100	112

Two conclusions may be drawn from these experiments. Of the enzymes used, only pepsin digests lysozyme at a rate measurable under the conditions of the experiment, and no proteins that are digested by the enzymes used, other than pepsin, were present in measurable quantities. Only small amounts of inactive, pepsin-digestible protein might be regarded as

[2] Unfortunately the activity after dialysis was not determined, but since lysozyme does not pass through the membranes it appears permissible to ascribe the activity measured before dialysis to the undialyzable fraction.

having been present, since no increase in activity of the undigested material was apparent. This conclusion is valid, however, only if the rate of digestion of the inactive material is greater than that for lysozyme.

Since many proteins are sensitized to enzyme action by heat, experiments were carried out to determine whether the protein or proteins in the lysozyme preparation could be thus sensitized and a differential digestion then be obtained.

TABLE IV

Hydrolysis of Lysozyme Preparation by Proteolytic Enzymes

Enzyme	Assay	Solids	Destruction of activity	Activity of undigested material
	total units	*gm*	*per cent*	*units per gm.*
Starting material	80	0.26		305
Bacterial proteinase	72	0.22	10	327
Mold proteinase	72	0.22	10	327
Trypsin	79	0.24	1	322
Pepsin	35	0.12	56	305
Papain	74	0.25	7	302

TABLE V

*Stability of Lysozyme to Heat**

Time	Assay	Loss	Loss
min	*units*	*units*	*per cent*
0	50.5		
5	50.7	0	0
10	46	4.5	9
15	44	6.5	13
25	40	10.5	21
50	30	20.5	40
80	20.5	30	60

* Heated in HCl (pH 3.0) at 96°.

Lysozyme has been reported to be stable to heat in acid solution but very labile at alkaline reactions. Before proceeding with the digestion experiments, we determined the effect of heating alone on the activity of lysozyme. Lysozyme powder at pH 2.8 was dissolved in distilled water and heated on a steam bath at a temperature of 96°, and samples were removed for analysis at regular intervals. While the rate of destruction was slow, a progressive decrease in activity was found which amounted to a 60 per cent loss after 80 minutes of heating (Table V).

A lysozyme solution similar to that used for the preceding experiment was heated until a destruction of 40 per cent had taken place. The solution was then treated with the various enzymes in the same manner as described for the unheated material. The results are presented in Table VI. Apparently the rate of digestion by all the enzymes had been markedly accelerated. After removal of the digested part by dialysis, however, the activity of the undigested remainder was the same as that of the original unheated material with one exception; that is, when papain was used. In this case the unit activity was reduced, which may mean that digestion of the material, inactivated by heat, was not complete or the products of digestion were not completely dialyzable. In no case was there any indication of a differential digestion resulting in increased activity of the undigested active preparation. It is also evident from these experiments that some change in the lysozyme molecule is induced by heating which does not disrupt the structure necessary for lytic activity but which does sensitize the molecule to enzyme action.

TABLE VI

*Hydrolysis of Heat-Treated Lysozyme Preparations by Proteolytic Enzymes**

Enzyme	Assay	Solids	Enzymic destruction of activity†	Activity of undigested material
	units	*gm*	*per cent*	*units per gm*
Starting material	46	0.16		293
Heated material	27	0.16	41 (By heat)	171
Bacterial proteinase	22.3	0.08	18	280
Mold proteinase	17.6	0.06	35	293
Trypsin	9.1	0.03	66	303
Pepsin	0.2	0.02	99	
Papain	16.5	0.07	39	236

* Heated in HCl (pH 3) at 98° for 90 minutes.

† Calculated as per cent of activity after heating.

Electrodialysis of Lysozyme Solutions—Solutions of lysozyme which had been dialyzed against distilled water were further freed from electrolytes by electrodialysis. The electrodialysis was carried out at 1° for approximately 18 hours, or until no appreciable change in conductivity of the solution took place. The pH, which was approximately 7.0 at the beginning, had risen to approximately 9.5 at the end of the experiment, and some precipitation of the solute took place. With adjustment of the electrodialyzed solution to pH 10 to 10.5, heavy precipitation resulted. At pH 11 resolution of the precipitate was apparent, and was complete at approximately pH 11.5. At pH 10.8, which appeared to be the point of minimum solubility, 6 mg. per ml. remained in solution, whereas, at pH 9 and 11.5, 150 mg. per ml. readily dissolved. No difference between the lytic activity of the precipitates and that of the material remaining in solution in the isoelectric region could be demonstrated. Therefore, no evidence for the presence of more than one component was found.

Longsworth, Cannan, and MacInnes (9) have reported that electrophoretic analysis of egg white proteins reveals the presence of one and only one component with an isoelectric point above pH 7.0. This component was designated by them as G_1. The average amount of this component comprised 2.8 per cent of the egg white proteins, whereas the average yield obtained in our preparations was 2.5 per cent of the proteins present in egg white. While the mobility of G_1 was not determined by Longsworth *et al.* above pH 7.8, extrapolation of the mobility curve shows an isoelectric region around pH 10.5 to 11.0, which is in close agreement with the isoelectric point of the protein contained in our lysozyme preparations. These facts point strongly to the identity of G_1 and lysozyme.

Electrophoretic Behavior and Isoelectric Point of Lysozyme—Several purified lysozyme preparations have been analyzed electrophoretically by the Tiselius method within the pH range of 4.5 to 11.8 and at an ionic strength of 0.1. In all of these analyses one component comprising more than 95 per cent of the total gradient was present and in several the material appeared quite homogeneous. In most of the analyses the ascending boundary appeared homogeneous, while in one or two trace components could be detected. The descending boundary in many of the analyses spreads considerably, beginning abruptly at the leading edge, sometimes with a small spike. It was in these spreading boundaries that evidence of the presence of trace components was chiefly observed (see foot-note to Table VII and Fig. 2). The conclusion seems justified that the lysozyme preparations are essentially electrophoretically homogeneous and may consist of quite pure protein. The results of all analyses are given in Table VII.

The mobilities of lysozyme approximate the values reported for the globulin component of egg white designated G_1 by Longsworth, Cannan, and MacInnes (9), as shown in Fig. 3. In the acid buffers the mobilities were lower than reported for G_1, but in Analyses 1 and 2 of Table VII the sharp leading edge coincides with this reported mobility. Phosphates depress the mobilities of lysozyme and G_1 from the values in buffers containing only monovalent ions to approximately the same extent. In the media near pH 7.8, two different samples were found to have mobilities closely similar to that reported for G_1, although differing in mobility from the lysozyme preparation reported by D. Moore in an article by Meyer ($+6.75 \times 10^{-5}$ sq. cm. per volt second at pH 7.80) (10).

The properties of G_1 at higher pH were not discussed by Longsworth and his associates, except for the statement that an isoelectric point was not

TABLE VII

Electrophoretic Analyses of Lysozyme Preparations

Analysis No.	Concentration, per cent protein	pH	Buffer composition*	Buffer molarity	Mobility of falling boundaries†	
					Principal	Trace
1	1	4.47	HA	0.15	+6.33	+7.6,‡ +5.1, +1.8
			NaA	0.1		
2	1	5.5	HA	0.017	+6.2	+6.9‡
			NaA	0.1		
3	1	6.03	HC	0.01	+5.3§	
			NaC	0.01		
			NaCl	0.09		
4	1	6.5	NaH_2PO_4	0.042	+3.6	+4.6‡
			Na_2HPO_4	0.019		
5	0.7	7.73	HV	0.01	+4.6	
			NaV	0.01		
			NaCl	0.09		
6	1	7.76	HV	0.01	+4.49	+5.9,‡ +3.7‖
			NaV	0.01		
			NaCl	0.09		
7	1	9.7	NH_4OH	0.02	+3.13	+3.27‡
			NH_4Cl	0.02		
			NaCl	0.08		
8	1	10.0	G	0.02	+3.8‡	
			NaOH	0.01		
			NaCl	0.09		
9	0.5¶	10.0	E	0.02	+3.22	−3.04
			HCl	0.01		
			NaCl	0.09		
10	0.5¶	10.7	E	0.1	+1.15**	
			HCl	0.01		
			NaCl	0.09		
11	1	11.80	NaOH	0.01	−2.3**·††	
			NaCl	0.09		

* Acids and salts present in buffers used were as follows: A = acetate, C = dimethylarsonate (cacodylate), V = diethylbarbiturate (barbital, veronal), E = ethanolamine, G = glycine.

† Mobilities are reported in terms of 10^{-5} sq cm. per volt second, referred to 0° by multiplying the mobility at the temperature of observation by the viscosity of water at that temperature relative to the viscosity of water at 0°.

‡ In several analyses the falling boundary spreads considerably, beginning abruptly at the leading edge, sometimes with a small spike. The rising boundary was sharp in these cases, so that the spreading may be attributed to imperfect ionic adjustment of solution and buffer, and the spike may not represent an extra component.

§ The sample, isoelectrically precipitated, was apparently quite homogeneous in this medium, but in Analysis 6 showed trace components, as noted.

‖ Mobility of minor component estimated from rising side.

¶ Nearly saturated solution.

** Apparently homogeneous, but the total displacement during the time available for analysis was insufficient to allow as conclusive a test as in other instances.

†† Analysis of the solution for biological activity and nitrogen content indicated that the lysozyme was stable under the conditions of the experiment.

observed. We found that lysozyme moved toward the anode in a medium of 0.01 N NaOH and 0.09 N NaCl (pH 11.80) in which its biological activity

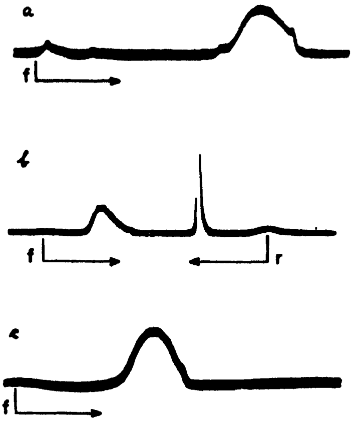

FIG. 2. Svensson-Philpot patterns of lysozyme preparations analyzed at 0.027 ampere per sq. cm. (*a*) Analysis 1, Table VII, after 12,000 seconds; (*b*) Analysis 2, Table VII, after 3000 seconds; (*c*) Analysis 5, Table VII, after 14,000 seconds.

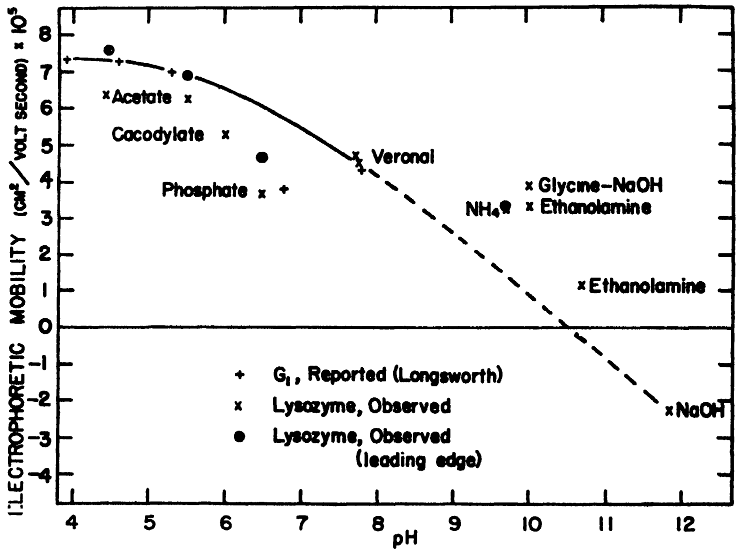

FIG. 3. The electrophoretic mobility of lysozyme as a function of pH, compared with that of the globulin G_1.

remained constant. Extension of the published graph to the mobility value at this pH indicates an isoelectric point near pH 10.5. However, the mobility of lysozyme in buffers containing ammonia, glycine, or ethanolamine, between pH 9.5 and 11, is regularly toward the cathode and faster than indicated by this extension of the trend of values at lower pH. The isoelectric point in these media seems to be near pH 11.0. This circumstance suggests complex formation with the amino constituent of the medium, just as the deviation with phosphates suggests phosphate binding.

The correspondence of the electrophoretic and chemical properties of the highly purified lysozyme with those of the globulin G_1 indicates that they are very similar and in fact may be identical.

Sedimentation, Diffusion, and Osmotic Pressure Measurements with Lysozyme—Solutions of the purified lysozyme were studied in an air-driven ultracentrifuge, a Lamm-Polson diffusion cell (11), and in a modified Hepp osmometer (12, 13).[3] The measurements indicated that the lysozyme preparation is homogeneous and that lysozyme falls among the lowest weight proteins listed by Svedberg (14).

From the sedimentation rates measured at lysozyme concentrations of from 1.5 to 0.5 per cent, in 0.15 M sodium chloride, values at $S_{20,\,w}$ were found to be about 1.9 Svedberg units. The average diffusion constant, $D_{20,\,w}$, calculated from the maximum scale line displacement and area, and by the method of successive analysis ((11) Equation 50) is 11.2×10^{-7} at 1 per cent lysozyme concentration. Partial specific volume measurements have not been made, but if we assume values from 0.70 to 0.75 we can calculate molecular weights from 14,000 to 17,000 from the sedimentation and diffusion constants obtained for this material.

The osmotic pressures were determined by extrapolating to zero time measurements with cellophane membranes (No. 300), through which lysozyme diffuses slowly. The pressures obtained were extrapolated to zero concentration of lysozyme by a plot of the ratio of pressure to concentration *versus* concentration. The measurements made at two concentrations of lysozyme gave the value of the ratio of pressure to concentration equal to 151. From this value a molecular weight of 17,500 is obtained.

Since partial specific volumes were not determined and osmotic pressure measurements were made with a slightly permeable membrane, the values for the molecular weight of lysozyme must be considered as approximate. However, they are in agreement with each other and also with the molecular weight of 18,000 given by Abraham (2).

Crystallization of Lysozyme—Lysozyme has been obtained in crystalline form. Crystallization has been successful under a wide range of pH values, ranging from the isoelectric region (pH 10.8) to 3.5. Two factors contribute to the ease of crystallization of lysozyme. It has a relatively high positive temperature coefficient of solubility, and the solubility of the amorphous material is 3 or 4 times as high as that of the crystalline form.

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[3] Davis, B. D., personal communication.

Crystallization at pH 4.5 was carried out in the following manner. 4 gm. of isoelectric protein, which had been dissolved in dilute acetic acid at pH 6 and dried in the frozen state, were dissolved in 60 cc. of 0.2 M acetate buffer at pH 4.5, containing 5 per cent of sodium chloride. The solution was allowed to stand at room temperature, when well defined crystals were deposited as shown in Fig. 4. Only 15 mg. per ml. of the original material remained in solution after crystallization for 16 hours. The rate of crystallization may be markedly increased by lowering the temperature from 21° to 4°.

Crystallization at the isoelectric region (pH 10 to 11) was accomplished by agitating an excess of the isoelectric protein with saturated sodium chloride adjusted to pH 11 with NaOH. The amorphous material partially dissolved, and as a result of the lower solubility of the crystalline product, crystals were deposited. When the process was continued long enough, all of the excess amorphous solid was changed to the crystalline state. The crystals so formed were very thin rectangular plates.

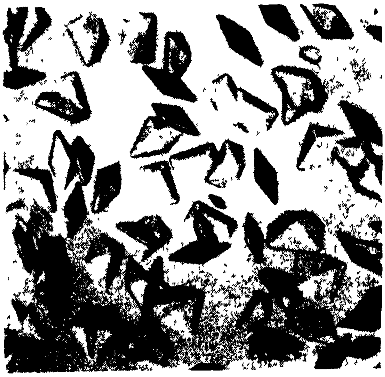

FIG. 4. Crystalline lysozyme (120 ×). Crystallized from 0.2 M acetate buffer, pH 4.5, containing 5 per cent NaCl.

Crystallization from ammonium sulfate solutions (0.4 M) buffered at pH 5.0 with acetate, or at pH 7.0 with phosphate, resulted when the temperature was lowered from 21° to 4°. Crystallization was also effected at room temperature from 1.4 M ammonium sulfate solutions acidified to pH 3.5 with sulfuric acid. The activities of the various crystalline preparations were in all cases similar to those of the amorphous material.

The crystalline forms obtained by the procedures outlined above seem to vary with the pH at which crystallization is carried out and with the acid used in effecting solution of the isoelectric material. Since lysozyme is a very basic protein, it seems that the crystal form of its salts with various acids may vary. This matter is being further investigated and the results will be reported later.

DISCUSSION

It is difficult if not impossible to compare the activity of our preparations with those reported by other investigators. Meyer *et al.* (4) stated that their preparations contain from 2 to 6000 units per mg. The unit is defined as the smallest amount causing complete lysis of *Micrococcus lysodeikticus* in a serial dilution test. The quantitative yield of active material is not given. Abraham (2) reported Robert's preparation (3) to be an improvement over that of Meyer *et al* (4) and to behave as a homogeneous protein in the ultracentrifuge. Robert's unit is similar to that of Meyer *et al.*, and the activities are likewise incapable of translation, since percentage yield and activity of egg white are not given Also, in our preliminary work with assay methods we found that the susceptibility of *Micrococcus lysodeikticus* varied many fold from day to day and from culture to culture, and hence found it necessary to use dead organisms in order to carry out quantitative work.

Abraham and Robinson reported in a short note in 1937 (15) that crystalline material was obtained from a lysozyme preparation which was prepared by Robert's method (3). This preparation appeared to be homogeneous in the ultracentrifuge, but Abraham (2) was able to separate two fractions of different solubilities and activities. Crystallization was induced by evaporation of a 0.05 N acetic acid solution *in vacuo* over aqueous KOH. 2 years later, however, Abraham (2) reported that he had not been able to obtain sufficient crystalline material for chemical examination. The fact that we have been able to obtain no evidence for the presence of two substances of varying activities and the ease of crystallization of our purified materials indicate that the two preparations may not be identical.

The facts presented in this paper indicate that lysozyme and the globulin called G_1 by Longsworth *et al* are identical. The amounts present, the isoelectric region, and the mobilities are all in approximate agreement. It appears unlikely from available information that more than one substance with these characteristics is present in egg white in the amounts found for G_1 and lysozyme.

While this paper was in preparation, two papers (10, 16) appeared dealing with the relationship of biotin, avidin, and lysozyme. In the preparations used in the work reported in these papers it seems that avidin activity and lytic activity were correlated, and it was intimated that lytic activity may depend on the presence of avidin and biotin. In preliminary experiments with the pure lysozyme preparations we have been unable to obtain any indication that avidin and biotin are concerned in the lytic activity of lysozyme. These experiments will be reported more fully in a later communication.

SUMMARY

1. A method for the isolation of lysozyme from egg white, in high yield and in essentially pure form, has been developed. The method depends on the (*a*) adsorption of lysozyme on bentonite (a montmorillonite clay), (*b*) elution of inactive contaminating proteins from the clay by successive washings with phosphate buffer (pH 7 to 8) and 5 per cent aqueous pyridine, and (*c*) elution of the active material with pyridine-sulfuric acid solution at pH 5.0. The eluate is dialyzed and dried in the frozen state. A white powder is obtained containing 85 to 90 per cent of the lysozyme contained in the egg white.

2. The lysozyme preparations have been shown to be essentially pure by salt fractionation, by their behavior toward enzymes, and by electrophoretic and sedimentation studies.

3. Lysozyme is a basic protein of low molecular weight (about 17,000). It is isoelectric at some point between pH 10.5 and 11.

4. The purified substance is stable in acidified solutions and relatively stable also in alkaline solutions. At pH 11.5 no loss of activity could be detected over a period of 5 to 6 hours.

5. Lysozyme has been prepared in crystalline form. Crystallization has been effected at the isoelectric region (pH 10.8), at pH 7.0, and in acid solutions (pH 3.5 to 5.0). The crystal form appears to vary, depending on the pH of crystallization and the acid used in dissolving the protein.

6. It seems probable that lysozyme and the substance termed G_1 by Longsworth *et al.* are identical.

We are very grateful to Dr. J. L. Oncley, Department of Physical Chemistry, Harvard Medical School, Boston, Massachusetts, and Dr. A. Brown, Laboratory of Physical Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts, for carrying out the sedimentation, diffusion, and osmotic pressure measurements reported in this communication. We are also indebted to E. Wolford and F. T. Jones of the Western Regional Research Laboratory for the preparation of the *Micrococcus lysodeikticus* organisms for test purposes and the preparation of the photomicrograph respectively.

BIBLIOGRAPHY

1. Fleming, H., *Proc. Roy. Soc. London, Series B*, **93**, 306 (1922).
2. Abraham, E. P., *Biochem. J.*, **33**, 622 (1939).
3. Roberts, E. A. H., *Quart. J. Exp. Physiol.*, **27**, 89 (1937).

4. Meyer, K., Thompson, R., Palmer, J., and Khorazo, D., *J. Biol. Chem.*, **113**, 303 (1936).
5. Buyanovskaya, I., and Jermol'eva, Z. W., *Acta med. U. R. S. S.*, **1**, 248 (1938).
6. Boasson, E. H , *J. Immunol.*, **34**, 281 (1938).
7. Wolff, L. K., *Z. Immunitätsforsch. u. exp. Therap.*, **50**, 88 (1927).
8. McMeekin, T. L., *J. Am. Chem. Soc.*, **61**, 2884 (1939).
9. Longsworth, L. G., Cannan, R. K , and MacInnes, D. A., *J. Am. Chem. Soc.*, **62**, 2580 (1940).
10. Meyer, K., *Science*, **99**, 391 (1944).
11. Lamm, O., *Nova acta reg. soc. sc. Upsaliensis*, **10**, No. 6 (1937).
12. Hepp, O., *Z. ges. exp. Med.*, **99**, 709 (1936).
13. Peters, E., and Saslow, G., *J. Gen. Physiol.*, **23**, 177 (1939).
14 Svedberg, T., and Pedersen, K. O., The ultracentrifuge, Oxford (1940).
15. Abraham, E. P., and Robinson, R , *Nature*, **140**, 24 (1937).
16. Laurence, W. L., *Science*, **99**, 392 (1944).

THE ESTIMATION OF TRYPTOPHANE IN HUMAN URINE*

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In the pursuance of our studies on the metabolism of tryptophane in man, we were faced with the need of a satisfactory method for the estimation of tryptophane in the urine. The literature revealed only descriptions of several qualitative urine tests (1, 2) which are not amenable to quantitative elaboration. Unsuccessful experiences with various other techniques turned us to attempt the adaptation to the urine of the tryptophane method described by us (3) for protein hydrolysates. The particular advantage of this method lies in the fact that the color reaction (modified Jolles) involved is specific for the indole nucleus and therefore not subject to interference from histidine, tyrosine, and phenols, as is the case with all colorimetric techniques based on oxidation or oxidation-aldehyde condensation reactions of tryptophane.

In the adaptation of our method for the estimation of tryptophane to the urine, two factors not incident to its application to protein hydrolysates had to be considered: (*a*) interference from some non-tryptophane indole derivatives, a difficulty which besets all of the available colorimetric tryptophane assays, and (*b*) low sensitivity of the modified Jolles reaction relative to the tryptophane content of normal urine. The difficulties arising from (*a*) were resolved by the finding that the non-tryptophane indoles which might occur in the urine are readily and quantitatively removed by ether extraction without loss of tryptophane. The augmentation in tryptophane concentration necessitated by (*b*) was found to be best achieved by precipitation of the amino acid with acid mercuric sulfate (4). The desirability of removing some of the other mercury complex-forming nitrogenous bases of the urine, the presence of which was indicated by initial experiments, was accomplished by an ionic exchange reaction with permutit (5). This process was found not to entail an appreciable loss of tryptophane. The interposition of this isolation procedure also involved the consideration and investigation of the correction for the solubility of the tryptophane-mercury complex. This was found to be equivalent to 0.78 mg. of tryptophane per 100 cc. of urine. It was also observed that other indole substances are precipitated by acid mercuric sulfate, so that the use of this reagent does

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not improve the specificity of tryptophane methods (6) for the analysis of biological preparations containing non-tryptophane indole compounds.

Experiments are presented herewith to demonstrate the suitability of our procedure for the estimation of tryptophane in the urine. The critical chemical tests received supplementary support by observations that ingestion of *l*- or *dl*-tryptophane by normal adult humans resulted in a prompt elevation of the tryptophane level in the urine. The study of seven normal male adults on normal diets revealed that 226 to 336 mg. of tryptophane are excreted daily and that the output bears a fairly constant ratio to the body weight.

EXPERIMENTAL

Reagents[1]—

Acid mercuric sulfate. 10 gm. of mercuric sulfate are dissolved in 100 cc. of 5 per cent (by volume) sulfuric acid solution.

Potassium persulfate, 1 per cent solution.

Sodium nitrite, 3 per cent solution.

Thymol solution. 1 gm. of thymol, U. S. P., is dissolved in 100 cc. of 95 per cent ethanol.

Acetic acid. Glacial and a 10 per cent solution.

Sulfuric acid, 50 per cent solution.

Acid reagent. 60 cc. of 40 per cent trichloroacetic acid are mixed with 40 cc. of concentrated HCl.

Tryptophane standard. 100 mg. of *dl*-tryptophane (Merck, 13.60 per cent N found) are dissolved in 100 cc. of distilled water. Since quantitative transfer of this substance is difficult, it is recommended that the amino acid be weighed directly in the volumetric flask. Solutions of tryptophane have been found to show appreciable deterioration within 1 week, even when stored in the refrigerator at 4°.

Procedure

Preservation of Human Urine—24 hour specimens were collected in 2 liter wide mouth brown bottles containing 50 cc. of 15 per cent HCl (by volume) and 1 cc. of 10 per cent alcoholic thymol and were made to a uniform volume of 2 liters before removal of the sample for tryptophane determination. Tests have shown that decomposition of tryptophane proceeds rapidly under these conditions, so that the assay must be carried out immediately at the termination of the collection period. Preservatives were not used for fractional specimens, since in these instances the determinations were made immediately.

Method—Description of the method will be subdivided into two sections:

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[1] Merck "reagent" grade chemicals were used throughout.

the isolation technique, which is performed only when the specimen is suspected to contain indole compounds other than tryptophane or to contain the amino acid in minute quantities, *e.g.* urine; and a colorimetric technique, which is applied to samples derived from the isolation operation or specimens which are free from indole derivatives other than tryptophane and contain 1 to 2 mg. of the amino acid per cc. of solution, *e.g.* protein hydrolysates.

Tryptophane Isolation Technique—50 cc. of urine are passed at the rate of 1 drop per second through a column of 10 gm. of activated permutit (5) contained in a 150 × 15 mm. (inside diameter) calcium chloride tube plugged with coarse glass wool and fitted with a short piece of rubber tubing and a screw pinch-cock to regulate the flow. When this is properly done, the filtrate should give a negative reaction with Nessler's reagent. To 20 cc. of the treated urine is added 1 cc. of 50 per cent sulfuric acid and the mixture is shaken in a separatory funnel with 20 cc. of ether, U. S. P., to remove any non-tryptophane indoles which may be present. (Owing to the formation of emulsions, the use of chloroform is not recommended.) The aqueous fraction is run into a 50 cc. graduated conical bottom centrifuge tube which contains 10 cc. of acid mercuric sulfate reagent. The mixture is refrigerated at 4° for 2 hours; then centrifuged for 10 minutes at 3000 R.P.M. The supernatant solution is decanted and the precipitate is dissolved in a minimum amount of acid reagent; usually 1 cc. is sufficient. The volume of this solution is adjusted accurately to the 5 cc. mark of the centrifuge tube with distilled water, or so that 1 cc. of the final solution contains approximately 1 mg. of tryptophane.

Colorimetric Technique—Two 2 cc. aliquots of the final solution derived from the isolation procedure in graduated Klett-Summerson colorimeter tubes are treated with 0.3 cc. of 3 per cent sodium nitrite and 0.1 cc. of 10 per cent acetic acid and the mixtures are shaken intermittently for 10 minutes. There are then added in succession 0.3 cc. of 1 per cent potassium persulfate, 0.5 cc. of thymol solution, and 5 cc. of acid reagent, with thorough mixing after each addition. The tubes are placed at once in a boiling water bath for 5 minutes and then cooled in a ice bath for 5 or 10 minutes. During the cooling, the reaction mixture will have separated into two layers, an almost colorless layer above and a red layer of ethyl trichloroacetate below. All but approximately 0.3 cc. of the upper layer is removed by aspiration through a capillary pipette at the water pump; then the colored layer is diluted to the 5 cc. mark with glacial acetic acid. The resulting color is read in the Klett-Summerson photoelectric colorimeter with a No. S-54 filter and compared with that of a similarly treated tryptophane standard (1 cc.). A reagent blank is also run and its color reading is subtracted from the readings of the unknown and standard before proceeding with the calculations.

Calculations—

U = colorimeter reading of unknown

T = " " " 1 cc. of tryptophane standard

RB = " " " reagent blank

Solubility correction = 0.78 mg. of tryptophane per 100 cc. of urine, or volume in cc. × 0.0078 mg.

Then

$$\text{Mg. tryptophane in sample} = \left(\frac{U-RB}{T-RB} \times \frac{5}{2} \times \frac{\text{volume in cc.}}{20}\right) + (\text{volume in cc.} \times 0.0078)$$

Results

The suitability of the modified Jolles reaction for the estimation of tryptophane was determined by submitting varying amounts of the amino acid directly to the colorimetric procedure. The results of these tests are shown in Fig. 1 and, since it appears that Beer's law does not apply strictly, the use of a calibration curve or standards within the range of the unknowns is recommended.

Although the modified Jolles reaction was previously found not to yield a red color with any of the known amino acids other than tryptophane, the behavior of other indole compounds which might occur in the urine remained to be tested. For this purpose authentic specimens of available indole derivatives were dissolved in suitable alcohol-water mixtures and the color reaction was carried out (*a*) directly on the test solution as described under the colorimetric technique, (*b*) after precipitation of the indole substances as described under the isolation technique, and (*c*) after shaking the test solution with an equal volume of ether. Comparison of the tryptophane equivalents of these compounds obtained by the three techniques indicates that, whereas the interference from the non-tryptophane indoles tested would be completely obviated by a single ether extraction, the precipitation procedure would not be effective in suppressing their interference (Table I). It is to be noted further that indole, indoleacetic acid, and indolepropionic acid give a lavender tint, but that tryptophane gives a reddish orange color with this reaction. It was surprising to find that tryptamine and acetyl-*dl*-tryptophane do not yield a color. It was further observed that all of the indole substances tested yield a precipitate with the mercury reagent. Moreover none of these indole compounds responded positively to the indican test, thereby clarifying the statements of Sharlit (7).

Since a pure specimen of indican could not be obtained, evidence of its failure to interfere with the tryptophane color reaction was deduced from tests which showed that the removal of indican from the urine by chloroform or ether extraction did not diminish the tryptophane value of urine specimens as determined by our procedure (Table II). In addition, these experiments indicate that normal urines do not contain appreciable amounts of non-tryptophane indole derivatives which react positively with the

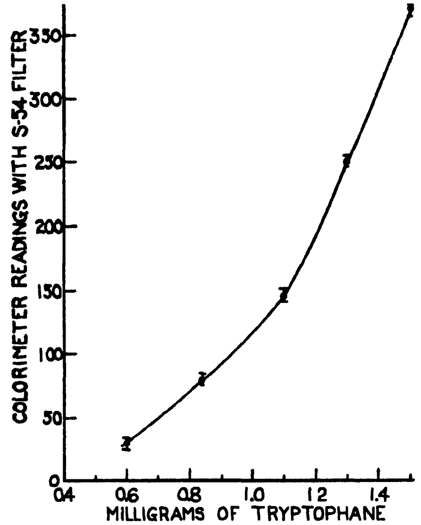

FIG. 1. Relation of color intensity to amount of tryptophane. Each point represents the average value of ten determinations. The bars above and below each point indicate the range of deviation of the readings. The colorimeter readings are given minus the reagent blank value (25 to 30 units).

TABLE I

Tryptophane Equivalents of Indole Compounds As Estimated by Application of Modified Jolles Reaction

Equivalents are based on the use of 1 mg. of each substance tested.

Substance	Direct color reaction (a)	After acid mercuric sulfate pptn. (b)	After single ether extraction (c)
	mg.	*mg*	*mg.*
Indole	12.0	4.7	0
Indoleacetic acid	11.4	3.5	0
Indolepropionic acid	0.5	0.2	0
Tryptamine	0	0	0
Acetyl-*dl*-tryptophane	0	0	0
dl-Tryptophane	1.0	0.96*	1.0

* Corrected for solubility of mercury complex.

modified Jolles test, so that it should ordinarily be possible to carry out the determination without previous ether extraction of the urine.

In order to secure urine samples containing adequate amounts of tryptophane for the color reaction, it was found necessary to prepare 6- to 10-fold concentrates. Although this can be accomplished by distillation *in vacuo*, precipitation of the amino acid as the mercury complex proved to be a more convenient and effective means of obtaining suitable samples for the determination. This latter approach was greatly facilitated by the observation that the requisite amount of reagent and resultant precipitate could be considerably reduced by removing the greater portion of mercury complex-forming N bases of the urine by adsorption on permutit. Detailed tests indicated that the introduction of this step does not entail any measurable loss of tryptophane. Still greater ease of operation and accuracy were achieved by the finding that, since the presence of mercuric ion does not affect the color reaction, the customary decomposition of the tryptophane-mercury complex by hydrogen sulfide could be replaced by solution of the compound in the acid reagent.

TABLE II

Effect of Removal of Indican on Tryptophane Value of Urine

Specimen	Extraction procedure	Tryptophane content	Indican	
			Total	Method
		mg	*mg*	
20 cc. Urine A	None	1.5	0.37	Sharlit (7)
Same	20 cc. ether, 1st	1.5	0.13	"
"	20 " " 2nd	1.5	0.03	"
20 cc. Urine B	None	3.3	0.29	Kumon (8)
Same	20 cc. $CHCl_3$	3.3	0.07	"

Recovery tests were performed at this point in the evolution of the method and revealed the need of a correction factor to compensate for the solubility of the tryptophane-mercury compound. Our data (Table III) indicate that the loss incurred under the conditions of the method is equivalent to 0.78 mg. of tryptophane per 100 cc. of urine. This figure could not be reduced by any variations in concentrations of mercuric sulfate or sulfuric acid. The need for such a correction for methods which interpose the precipitation technique was pointed out by Cary (9). He found a correction of 0.035 mg. of tryptophane per 100 cc. of reaction solution necessary under the conditions of his procedure. The discrepancy with our values may well be due to difference in characteristics of the media which Onslow (10) has demonstrated to affect the precipitability of amino acids by acid mercuric sulfate.

Further evidence of the validity of the method for measuring urinary tryptophane was adduced from human feeding experiments. For this purpose four fasting normal subjects were fed 2.0 gm. (0.01 M) of *l* or *dl*-tryptophane and given 240 cc. of water at zero hour and 120 cc. more at the end of the 1st and 2nd hours to maintain a uniform flow of urine for the period of the experiment. The urine was collected at 0, ½, 1, 2, and 3 hours and submitted to the analyses. The temporary tryptophanuria observed in both subjects leaves little doubt that the reaction is specific for tryptophane (Fig. 2). It is to be noted that, although the tryptophane output after ingestion of racemic and *l* (−)-tryptophane is not appreciably different, the feeding of the racemic variety results in the formation of an unidentified indole metabolite which fails to affect either indican or tryptophane values and yields indigo red on treatment with iodine (11).

Table III

Analytical Losses of Tryptophane Due to Solubility of Tryptophane-Mercury Complex in Human Urine

Conditions of tests, final volume 31 cc., temperature 4°, time 2 hours.

Urine specimen and tryptophane content	Tryptophane added	Tryptophane in composite sample		Tryptophane loss	
		Calculated	Found	Total for test	Calculated for 100 cc. urine
	mg	*mg*	*mg*	*mg*	*mg*
20 cc. Urine A; 2.27 mg.	0.91	3.18	3.08	0.10	0.50
Same	1.83	4.10	3.98	0.12	0.60
20 cc. Urine B; 2.81 mg.	3.26	6.07	5.86	0.21	1.05
Same	6.52	9.33	9.13	0.20	1.00
"	13.04	15.85	15.68	0.17	0.85
"	26.08	28.89	28.76	0.13	0.65
Average and average deviation					0.78 ± 0.19

Attention is also called to the interesting fact that only about 1 per cent of the tryptophane fed was recovered in the urine as excess above the normal output (Fig. 2). This observation is corroborated by other experiments in which the intake of 30 to 35 gm. of heterogeneous proteins, as lunch, was found to induce an over-all increase of only 6.0 mg. of tryptophane above the normal level in subject A (male) and no increase above the basal output in subject F (female). These findings signify that only a very small amount of the total urinary tryptophane can be attributed to postprandial spilling of the amino acid.

Measurements made by this method on the urines of seven normal adult males maintained on normal diets show that an average of 281 ± 30 mg.

of tryptophane was excreted daily, the amino N of which is equivalent to 3.9 ± 0.3 per cent of the total amino N. Calculations from these data show

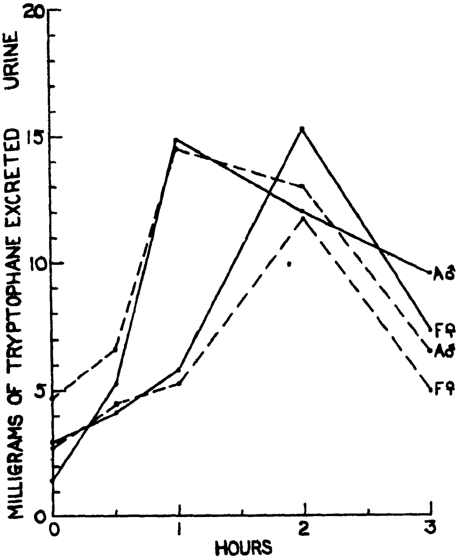

FIG. 2. Urinary output of tryptophane of two fasting subjects after administration of 0.01 M (2.0 gm.) of *l*- or *dl*-tryptophane. The solid line indicates excretion after ingestion of the *dl* form; the broken line after ingestion of the *l* variety. Subject A, male, 70 kilos; subject F, female, 60 kilos.

TABLE IV

Daily Tryptophane Output of Normal Adult Human Males on Normal Diet

Subject No	Body weight	Total amino N	Tryptophane output	$\frac{\text{Tryptophane}}{\text{Body weight}}$
	kg	*mg*	*mg*	*mg*
1	103.0	600	336	3.26
2	94.0	690	316	3.26
3	74.4	460	250	3.38
4	81.6	390	288	3.53
5	90.1	490	264	2.93
6	80.7	410	295	3.62
7	68.0	460	226	3.32
Average and average deviation...		500 ± 83	282 ± 30	3.33 ± 0.15

that the output of tryptophane appears to bear a fairly constant ratio to the body weight of the individual (Table IV). These subjects were subsequently submitted to various experimental but adequate diets for 60 days and the tryptophane measurements made over this period revealed the over-all average daily tryptophane output to be 3.28 ± 0.24 mg. of tryptophane per kilo of body weight. This is in good agreement with the value found for the normal diet period (Table IV).

DISCUSSION

Owing to the lack of specificity of the available methods, attempts to utilize the tryptophane content of various biological fluids as a diagnostic criterion in a variety of diseases have led to conflicting views on the value of this test (12). Most interesting of these studies are the reports on serum tryptophane in liver cirrhosis (13) and in pulmonary tuberculosis (14), cerebrospinal fluid tryptophane in tuberculous meningitis (15), and tryptophanuria in mental diseases (2). It would seem possible that clarification of the significance of the tryptophane test in these and other diseases could be effected by the application of the method presented here.

The finding of large amounts of tryptophane in the urine naturally raises questions as to its origin, whether *extrinsic*, due to a temporary hypertryptophanemia following ingestion of food, or *intrinsic*, due to normal tissue breakdown processes. Evidence for the latter possibility is deduced from the observations that (*a*) the normal tryptophane output is only slightly elevated as the result of the intake of tryptophane or food, (*b*) the rate of excretion remains fairly constant throughout the day, and (*c*) the loss is relatively constant as measured by the ratio of tryptophane to body weight. If this hypothesis is correct, the value for urinary tryptophane (3.3 mg. of tryptophane per kilo of body weight per day) would represent a minimum amount of the natural form (11) of this essential amino acid (16) which should be restored to the organism to maintain tryptophane equilibrium.

The fact that tryptamine, acetyl-*dl*-tryptophane, and indican do not yield a red color by this test is of particular significance to us in view of the fact that the unidentified indole metabolite of *d*-tryptophane produced by man apparently also fails to give a positive test by this reaction. However, other tests have shown that the *d*-tryptophane metabolite is not identical with either of these three compounds.

Finally, it is of interest to note that tryptophane was found to constitute the principal indole derivative present in the urine of twelve normal human adults (four females and eight males) examined intermittently for various indole compounds over a period of 2 months.

SUMMARY

A method for the estimation of tryptophane in human urine is described. Application of the procedure to 24 hour specimens of seven normal male subjects on normal diets has revealed that 281 ± 30 mg. of tryptophane are excreted daily and that the output approximates the value 3.3 mg. of tryptophane per kilo of body weight per day. The origin and physiological implications of urinary tryptophane are discussed.

BIBLIOGRAPHY

1. Fearon, W. R., *Dublin J. Med Sc.*, March 28 (1920).
2. Brice, A. T., *Arch. Int. Med* , **46,** 778 (1930); **49,** 978 (1932).
3. Albanese, A. A., and Frankston, J E , *J. Biol Chem* , **144,** 563 (1942).
4. Hopkins, J. G., and Cole, S W , *J Physiol* , **27,** 418 (1901–02).
5. Whitehorn, J. C , *J. Biol. Chem* , **56,** 751 (1923)
6. Folin, O , and Marenzi, A. D , *J. Biol Chem* , **83,** 89 (1929)
7. Sharlit, H., *J. Biol. Chem.*, **99,** 537 (1932–33).
8. Kumon, T., *Z. physiol. Chem.*, **231,** 205 (1934).
9. Cary, C A., *J. Biol. Chem.*, **78,** 377 (1938)
10. Onslow, H , *Biochem. J.*, **15,** 392 (1921)
11. Albanese, A. A., and Frankston, J E , *J. Biol. Chem.*, **155,** 101 (1944).
12. Re, P M , Acidos aminados, Buenos Aires, 780–813 (1940).
13. Jezler, A , and Mezey, K , *Klin Wochschr* , **10,** 1296 (1931)
14. Adler, F , *Beitr Klin Tuberk* , **84,** 291 (1934). Musso, E , *Gior. batteriol. e immunol* , **19,** 861 (1937).
15. Lichtenberg, H. H , *Am. J. Dis Child* , **43,** 32 (1932).
16. Holt, L. E., Jr., Albanese, A A., Brumback, J E , Jr , Kajdi, C. M., and Wangerin, D. M , *Proc Soc Exp Biol and Med.*, **48,** 726 (1941).

SPECTROPHOTOMETRIC STUDIES

XII. OBSERVATION OF CIRCULATING BLOOD IN VIVO, AND THE DIRECT DETERMINATION OF THE SATURATION OF HEMOGLOBIN IN ARTERIAL BLOOD*

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Uncertainty still exists concerning a fundamental point in hemoglobin physiology, the degree of saturation of arterial blood at sea level. Values for this quantity (per cent of oxyhemoglobin, HbO_2) in man, reported in the more recent literature (5–7), are as low as 93.0 per cent and yield an average of 95.0. Such values for *percentage saturation* have been used in deducing the *oxygen tension* (pO_2) of arterial blood, usually read off from previously determined oxygen dissociation curves which relate saturation to tension. On typical dissociation curves (8) 93.0 and 95.0 per cent HbO_2 correspond respectively to approximately 65 and 80 mm. of Hg of arterial pO_2. If these values are correct, a difference (ΔpO_2) of appreciable magnitude (average = 20 mm. of Hg) exists between alveolar and arterial oxygen tensions. This ΔpO_2 has been accepted as a physiological phenomenon, and has been explained by the hypothesis that "oxygen equilibrium is not attained until after passage through the lung capillaries" (9). It may be pointed out at once that this indirect procedure for obtaining the arterial pO_2 is not precise. The portion of the dissociation curve which applies to arterial blood at sea level is asymptotic; so that an increase in arterial saturation by 2 per cent (from 95 to 97 per cent) would markedly reduce, and an increase of 3 per cent (from 95 to 98) would practically erase the ΔpO_2 (8). Thus, the question whether the values of arterial saturation, as obtained by current methods, are too low becomes a vital one.

The usual gasometric technique (10) for determining the percentage of saturation of hemoglobin with oxygen is indirect. Two separate analyses,

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* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Pennsylvania. Permission for publication has been granted Preliminary reports were presented at the May 16, 1944, meeting of the Physiological Society of Philadelphia, and abstracts have appeared (1, 2). At the same meeting reports were made upon related investigations of sources of error in the gasometric determination of oxygen saturation by Roughton, Darling, and Root (3), and the determination of arterial oxygen tension by Comroe (4). The results of the independent investigations were mutually concordant.

oxygen content and *oxygen capacity*, are required. The oxygen content usually is determined fairly promptly after the blood, unexposed to air, is drawn, whereas an indefinite interval of time (often 1 hour or longer) may elapse before the analysis of oxygen capacity is performed upon an aliquot which has been equilibrated with air in a tonometer. Since blood is not an inert system, it may well be questioned whether the original sample and the subsequent aliquot are strictly comparable. This issue has ceased to be academic in view of reports (11–13) that normal blood may contain substances (other than HbO_2) which, after treatment with dithionite ($Na_2S_2O_4$), combine with CO, thereby increasing the CO-combining capacity. One such substance may be methemoglobin, MHb. It is known that MHb, when present in freshly drawn blood samples, may rather quickly revert to HbO_2 (through Hb) when the blood sample is allowed to stand (14). Thus in the usual analysis of percentage saturation of blood samples containing a small amount of MHb, the latter may change to HbO_2. Opportunity for reversion is small in the promptly performed estimation of oxygen content, but it is much greater in the longer interval involved in the oxygen capacity determination. Such a phenomenon could account for an oxygen capacity too high relative to the oxygen content, and as a result the value of percentage saturation would be too low. Factors of this type have been investigated and discussed fully by Roughton and his associates (3).[1,2]

In seeking additional information upon the saturation of hemoglobin we have turned to the spectrophotometric analysis of arterial blood. The optical technique is particularly appropriate for the direct quantitative determination of two or more species (in the present work, HbO_2 and Hb) in a solution (16, 17). The Drabkin and Austin special cuvette of 0.007 cm. depth and chamber volume of 0.021 ml. (18) was utilized. This cuvette was designed for and has been used successfully in the measurement of absorption spectra of blood hemolyzed by saponin and of concentrated solutions of hemoglobin unexposed to environmental gases (16, 18). The technique has been extended to a study of turbid systems such as whole, unhemolyzed blood (19) and the measurement of mixtures of intracellular HbO_2 and Hb in a study of the penetration of $Na_2S_2O_4$ and ascorbic acid into erythrocytes (20). A cuvette of similar design (but of about twice the depth) has been adapted by Lowry, Smith, and Cohen (21) to a photoelectric, filter photometer, for the estimation of HbO_2-Hb in samples of

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[1] The mechanism of MHb reversion has been studied by one of us (D. L. D.), and will be published separately. The study revealed that the disappearance of MHb from blood is inhibited by fluoride and iodoacetate, and that the inhibition by fluoride can be removed by pyruvate. Reference may be made to Kiese's recent report (15) upon the mechanism of MHb reduction.

[2] Roughton, F. J. W., Darling, R. C., and Root, W. S., to be published.

cutaneous blood, hemolyzed and slightly diluted by the addition of a solution of saponin and ammonia. In this communication we shall report (1) the successful performance of continuous spectrophotometric observation of circulating arterial blood *in vivo* in the dog, and (2) the determination of hemoglobin saturation in dog and man by direct spectrophotometry on undiluted arterial samples, hemolyzed without exposure to air, and measured within 2 to 3 minutes after withdrawal from the subject. The arterial saturation was found to be consistently of the order of 98 per cent in both sets of determinations. As far as we know, direct spectrophotometric observation of circulating blood has not been reported previously. The closest approach to the present technique is the ingenious work of Kramer and his colleagues (22, 23), who made photoelectric measurements upon transilluminated blood vessels, utilizing the difference in light transmission of HbO_2 and Hb over a broad spectral range of red and near infra-red. Outgrowths of Kramer's technique are the method of Matthes and Gross (24) and Millikan's oximeter (25).

Methods

The subjects of the experiments were six healthy, mongrel dogs weighing 8.6 to 12.5 kilos, and five young men, 17 to 23 years of age. In the latter group four were normal and one a controlled diabetic, maintained with insulin (subject R. C. L., Table II). Four of the subjects were nonsmokers; one (L. J. D., Table II) had abstained from smoking for a period of 3 days prior to the experiment, a precaution taken to avoid the presence of HbCO (3).[2] The dogs were in the postabsorptive state, and the human subjects were instructed to partake of only a very light breakfast, the arterial punctures being performed 4½ to 5 hours thereafter.

Spectrophotometric Observation of Circulating Blood—The 0.007 cm. cuvette (18) was filled with isotonic saline[3] and then introduced into the stream of one femoral artery of the dogs, which were anesthetized with nembutal (40 mg. per kilo intraperitoneally) and aligned on a board beneath the optical bench of the spectrophotometer. The arrangement is described in Fig. 1 and the accompanying legend. Clotting was prevented by previous intravenous injection of a 5 per cent solution (100 mg. per kilo) of du Pont's pontamine fast pink[4] (same as chlorazol fast pink 2B (26)) and

[3] A volume of approximately 0.2 ml. is required to fill the cuvette and its entry and exit capillaries. The saline is not essential, but is helpful in establishing immediate homogeneous blood flow after the circulation is diverted through the cuvette. It should be emphasized that in all the measurements upon hemolyzed (saponized) blood, the sample was admitted only into scrupulously clean and thoroughly dry cuvettes.

[4] The synthetic anticoagulant pontamine fast pink was found to have an absorption curve with a slight inflection at 562 mμ and a maximum at 521 mμ. The respec-

periodic injection (at approximately half hour intervals) of 0.5 ml. quantities of heparin. With heparin alone circulation through the cuvette could be maintained for only approximately 5 minutes before the flow was interrupted by deposition of fibrin in the chamber. The arrangement of connections (Insert 2, Fig. 1) from the artery to cuvette provided for arresting the flow through the chamber, when desired, and by-passing it through a second channel to the artery. This permitted periodic trapping of samples at different phases of the respiratory cycle, and the evaluation of the effect of this factor on hemoglobin saturation, disclosed in some of Kramer's studies (22, 23). To allow for rapid changes from room air to 100 per cent or 10 per cent oxygen, in these experiments a cannula was inserted in the trachea. The respiratory rate of the nembutalized animals was 10 to 12 per minute. Samples of blood were removed periodically for erythrocyte count and for independent spectrophotometric analysis of hemoglobin by the usual technique (27) with the 1 cm. cuvette upon diluted, hemolyzed blood as HbO_2, $Hb(HbO_2 + Na_2S_2O_4)$, and total pigment as cyanmethemoglobin, MHbCN.

Collection and Preparation of Hemolyzed Arterial Blood, Unexposed to Environmental Gases—To avoid the possibility of equivocal results due to use of pontamine fast pink and turbid systems, and to assure full precision of spectrophotometric measurement, the following procedure was adopted. Small tonometers of 6 to 15 ml. capacity, provided at each end with the usual double bore stop-cocks, permitting discard of the blood which first flows through the entry tube, were employed as collection vessels. A solution containing 30 mg. of oxalate and 50 mg. of saponin (Merck, purified)

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tive millimolar ϵ values at these wave-lengths are 10.9 and 20 4, with a molecular weight of 928 (based on the value for chlorazol fast pink (26)). It may be calculated, with a value of 8 per cent of body weight in gm. for blood volume in ml., that the original concentration of the dye in the blood in our experiments should be approximately 1.3 mM per liter. The concentration of total hemoglobin (Table I) is of the order of 10 mM per liter. When these respective concentrations were duplicated *in vitro* (*i.e.* dye added to drawn blood), the absorption spectra upon clear solutions (diluted 1:100) yielded results predictable from the contributions to absorption of the two components, dyestuff and HbO_2. On the other hand, blood samples withdrawn from the animals following injection of the dye, upon dilution to 1:100 for spectrophotometry by the usual technique in 1 cm. cuvettes, showed only a small influence on the absorption spectrum of HbO_2. In samples withdrawn early after injection of the dye the characteristic ratios of $\epsilon_{578 \text{ m}\mu}:\epsilon_{562 \text{ m}\mu}$ and $\epsilon_{542 \text{ m}\mu}:\epsilon_{562 \text{ m}\mu}$ were changed from 1.75 and 1.69 respectively to 1.70 and 1.62. As the experiment progressed (2, 3, and 4 hours after injection), restoration towards the normal ratio was observed. The water-soluble dye promptly spills over into urine, but the impression was gained that this accounts for only a fraction of that removed from the circulation. A precise study of the rate of disappearance of pontamine fast pink from the blood was not undertaken.

(quantities sufficient for approximately 4 ml. of blood, the size of the usual sample) was evenly air-dried on the walls of the tonometers. In this operation care was exercised to obtain a fine, thoroughly dry deposit with no evidence of trapping of air bubbles. The tonometers were then connected by means of clean pure gum tubing to small leveling bulbs and filled with

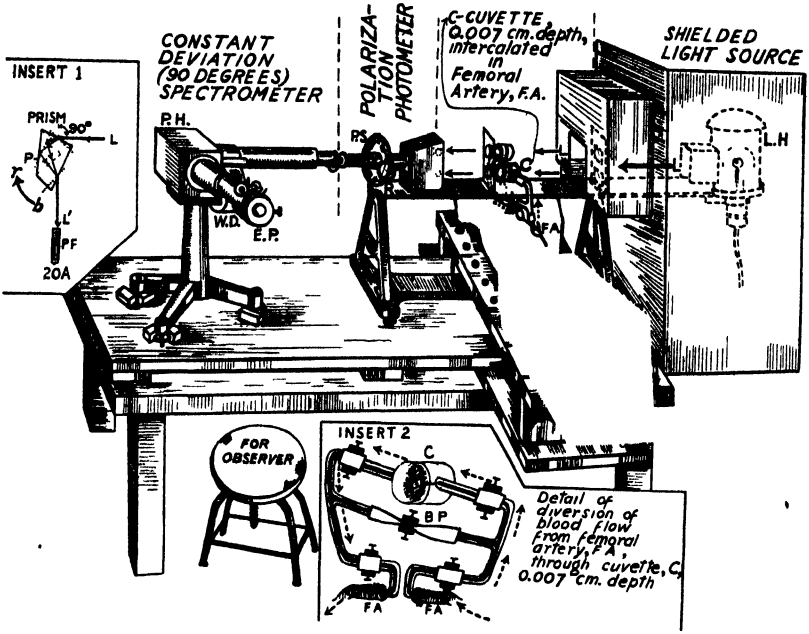
INSERT 1
PRISM
90°
L
P
r
b
L'
PF
20A
CONSTANT DEVIATION (90 DEGREES) SPECTROMETER
P.H.
W.D.
E.P.
POLARIZATION PHOTOMETER
PS
R
C-CUVETTE, 0.007 cm. depth, intercalated in Femoral Artery, F.A.
C
FA
SHIELDED LIGHT SOURCE
L.H
FOR OBSERVER
INSERT 2
C
BP
FA
FA
Detail of diversion of blood flow from femoral artery, F.A., through cuvette, C, 0.007 cm. depth

Fig. 1. Arrangement for continuous direct spectrophotometric observation of circulating blood *in vivo*. The alignment of the Bausch and Lomb spectrophotometer assembly, permanently set up in a dark room, is shown. The observer, reading through the eyepiece, *E P*, of the spectrometer, is completely shielded from the light, *L*, by enclosing the lamp housing, *L. H*, and accessory biprisms (which split the light into two parallel, optical paths) in an additional large box shielding. Insert 1, a rectangular monochromatic, *L'*, photometric field (of two halves), *P. F.*, of 20 A in width is defined by a diaphragm in *E.P.* Different wave-lengths are brought into position by rotation of a drum, *W. D.*, translated into rotation of the prism (housed within *P. H.*) from red, *r*, to blue, *b*, or vice versa, about the pivotal point, *P*. The photometer scale, *P. S.*, after matching the half fields, is read at *R*. Insert 2 illustrates the introduction of the Drabkin and Austin special cuvette into the femoral artery, *F. A.* The arrangement of clamps is shown. The passage of blood through the cuvette may be arrested and the circulation by-passed by closing the clamps at the entry and exit capillaries of the cuvette and opening the clamp, *B. P.*, in the by-passing channel. The connections at the clamp sites between the glass capillaries are of small bore, pure gum tubing.

thoroughly clean mercury[5] and were repeatedly evacuated to insure freedom from traces of trapped air. Before connection was made by means of a 2 cm. length of pure gum tubing to the cannula or needle in the artery, the tonometers were completely filled with mercury, including the entry tube. In the anesthetized dogs the blood was delivered through a glass cannula inserted in the femoral artery. In man (at rest, and supine) the arterial blood was obtained under local procaine anesthesia by the method of femoral puncture, by use of cut down lumbar puncture needles (with stilus), gage 19. With the stilus withdrawn, a slow, free flow of blood was provided. The needle was allowed to remain in the artery for periods of about 1 hour, during which time samples were drawn at intervals into the tonometer collection vessels, with the level of the mercury reservoir adjusted to exert only very slight negative pressure. In all cases the first flow of blood was discarded through the side tube of the tonometer. Samples in which suspicion existed of the possible trapping of minute bubbles of air were not used.

The blood samples were usually collected in three to four spurts, timed to synchronize with "end" inspiration or "end" expiration, kymographically recorded in both dog and man. By gentle shaking the blood sample was quickly hemolyzed. The blood in the entry tube of the tonometer was removed with a pipe-stem cleaner and replaced by the hemolyzed solution. After quick establishment of connection between tonometer and entry capillary of the dry, thoroughly clean special cuvette, the sample was transferred to the latter by adjusting the reservoir to very slight positive pressure. Enough of the hemolyzed sample was transferred to allow for appreciable overflow into the exit capillary of the cuvette. Since the volume of the chamber of the latter is 0.021 ml. and the volume of the capillary tubes approximately 10 times greater, the optical chamber of the cuvette is completely washed out, thereby assuring that the specimen within has been transferred without contamination with environmental gases. The overflow of blood in the capillary entry and exit tubes effectively seals the chamber, so that oxygenation of the contained sample will not occur for periods of time up to 1 hour (18). Since the element of time has been considered as possibly essential in the present problem, it should be stated that *spectrophotometric measurement was begun within 2 to 3 minutes and completed within 6 minutes after withdrawal of the blood from the subject.*

The remainder (about 3 to 3.5 ml.) of the hemolyzed blood was transferred from the tonometer to a flat weighing dish. After exposure to air in this container (in which drainage difficulties are not encountered (3)[2] portions were reread spectrophotometrically in the 0.007 cm. cuvette with and without addition of $Na_2S_2O_4$. An aliquot was diluted and determined in the 1 cm. cuvette as HbO_2 and, after conversion, as Hb and MHbCN.

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[5] In one instance failure to observe the elementary precaution of using scrupulously clean tubing and mercury resulted in the formation of appreciable amounts of changed pigment, recognized spectroscopically as MHb from the character of the absorption spectrum and the effects of addition of $Na_2S_2O_4$ (conversion to Hb), addition of KCN (conversion to MHbCN), and change in pH (17).

Spectrophotometric Technique, and Error of Method—For details of the technique readers are referred to a recent review (16) and to earlier papers in this series, particularly upon the spectrophotometry of turbid systems (19) and the method of Austin and Drabkin (17) for the determination of two or more species in a single solution. In the latter technique, here applied to mixtures of HbO_2 and Hb, accuracy is increased by measurements at several characteristic wave-lengths, rather than being limited to two spectral regions. In the present work calculations were based upon ϵ values[6] at 600, 578, 562, and 542 mμ, with the wave band or spectral interval limited to 2 mμ, and total pigment concentration determined by conversion to MHbCN[7] (27). Measurements were also made at wave-lengths of 630, 555 (maximum of Hb), and 505 mμ (an isobestic point in the absorption curves of HbO_2 and Hb). Readings at 505 mμ are not highly accurate, but can serve as a check upon total pigment concentration (Table I, last column). An example of the procedure and calculation employed is furnished in Fig. 2. At the characteristic wave-lengths, ϵ values must be known or must be determined for the individual species, HbO_2 and Hb. The summation of the total change in absorption, $\Sigma\Delta\epsilon$, at these wave-lengths between HbO_2 and Hb is designated Σ_T. The summation of the partial change, Σ_p, is the change in absorption from HbO_2 to the particular mixture, M, of the two pigments measured. The ratio, $r = \Sigma_p:\Sigma_T =$ *fraction of Hb*, and $1 - r =$ *fraction of* HbO_2. $(1 - r) \times 100 =$ *percentage saturation.*

Mean ϵ values have been established with sufficient precision for hemolyzed dog blood to be used as absorption constants. The mean ϵ values, obtained in the present experiments (recorded in Fig. 2), agree very closely with values previously obtained upon hemolyzed dog blood in the 0.007 cm. cuvette (18), and differ only very slightly from the absorption constants upon diluted hemolyzed blood, measured in the 1 cm. cuvette (27). Therefore, with saponized dog blood valid interpretations could have been obtained from measurements limited to the experimental sample, containing HbO_2 and Hb, by utilizing the previously established (18) ϵ values for individual pigments. However, a sufficiently large number of analyses was not available for the precise establishment of constants for HbO_2 and Hb in

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[6] As heretofore, our ϵ values are for a concentration of 1 mM per liter (in the case of hemoglobin, referred to an equivalent weight of 16,700) and a depth of 1 cm. Thus, $\epsilon = (1/(c \times d)) \times \log I_0/I$, where the concentration c is expressed in mM per liter, the depth d in cm., the original intensity I_0 is 1.0, and the intensity of transmitted light I is expressed as a fraction of unity.

[7] In the spectrophotometric determination of total pigment concentration upon aliquots converted to MHbCN, $\epsilon = 11.5$ at 540 mμ is used. This constant has been established for preparations from hemolyzed, washed dog erythrocytes (27). The same constant was used in the present measurements for hemolyzed, human blood. Evidence for the validity of this procedure will be furnished in a subsequent report.

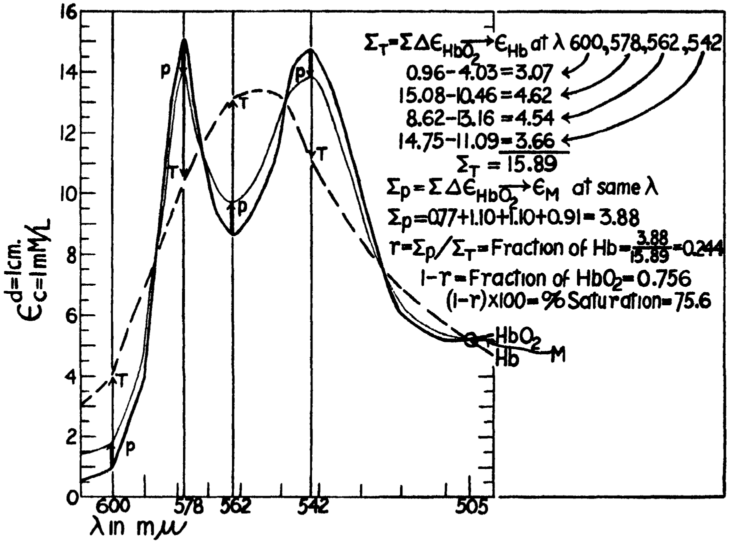

FIG. 2. Absorption spectrum curves obtained from measurements on hemolyzed (saponized) dog whole blood in the 0.007 cm. cuvette, with an example of the method used in the direct spectrophotometric determination of percentage saturation. Curve HbO_2 (heavy, solid line), based on mean ϵ values for fully oxygenated blood from six normal animals in the present study The absorption constants (mean ϵ values) for HbO_2, at the indicated characteristic wave-lengths used in the estimation of saturation, are given in the column headed by 0 96. Curve Hb (broken line), based on mean ϵ values upon aliquots of the samples used for Curve HbO_2, after deoxygenation by means of solid dithionite, $Na_2S_2O_4$ (0.1 mg. per ml.). The mean ϵ values, used in the estimation of saturation, appear in the column headed by 4.03. Curve M (light, solid line), sample from Dog 4, partially deoxygenated in a tonometer by CO_2 and N_2, total pigment concentration (as MHbCN) = 9.81 mM per liter. Appended data show that the curve represents a mixture of 0 756 HbO_2 and 0.244 Hb. The percentage saturation of the sample is therefore 75.6. For symbols, see the text under "Methods."

the blood of man. Hence the procedure was adopted of determining for each individual hemolyzed blood sample (1) the original absorption spectrum (of the portion transferred directly into the 0.007 cm. cuvette), (2) the spectrum after the remainder had been fully oxygenated (read undiluted in the 0.007 cm. cuvette, and also read diluted 1:100 in the 1 cm. cuvette as a check upon complete oxygenation), and (3) the spectrum of an aliquot treated with solid $Na_2S_2O_4$ (read both undiluted and diluted). The redetermination of the sample in the 0.007 cm. cuvette after procedures (2) and (3) yields the ϵ values for HbO_2 and Hb respectively, from which the values of $\Sigma\Delta\epsilon_{HbO_2 \rightarrow Hb}$, or Σ_T, are obtained for each sample. The individual Σ_T values (Table II, third column) were employed in the calculation of percentage saturation in the arterial blood of man. Results (or conclusions), however, would not be materially altered if the mean value $\Sigma_T = 16.38$ and the mean ϵ values for HbO_2 and Hb had been used. With the dog blood, mean ϵ values, obtained in the present work, which yielded mean $\Sigma_T = 15.89$, were used, since practically identical results were obtained by the individual treatment of each sample.

Based upon the deviation of ϵ values from the mean in the establishment of absorption constants, or the determination of hemoglobin concentration (as MHbCN), Drabkin and Austin (27) concluded that the accuracy of the spectrophotometric method was ±0.5 per cent, and greater in their hands than the accuracy attained in parallel analyses by the gasometric technique. In the determination of mixtures, under ideal conditions, practically as high accuracy was obtained as for individual pigments (17). In the present work the accuracy may be expected to be of a somewhat lower order, since analyses were carried out upon mixtures in which the proportion of one component is very small in comparison with the other. On the other hand the need for volumetric manipulations (measurement of volume of blood and its dilution) with their possible errors is eliminated in the use of the 0.007 cm. cuvette. A conservative estimate would be that the analytical error is within ±1.0 per cent, a value well beyond the agreement of analytical results such as Σ_T (in human blood) $= 16.38$, standard deviation $= 0.04 \pm 0.01$, and the concordance of results presented in Table I.

Results

Observations on Circulating Blood. General Findings—(1) A homogeneous circulation of the diverted blood flow through the cuvette chamber was maintained, permitting continuous visual measurements up to periods of 4 hours. (2) With thoroughly clean cuvettes the film of blood rapidly filled the entire cuvette chamber and uniform photometric fields were rapidly established and maintained. *Streaming effects were totally absent.* The gratifying absence of streaming or channeling, which would have vitiated the application of photometry, appears remarkable in view of the thinness of the chamber. the depth of which is approximately only 9 times the diameter of the red blood cell. (3) The passage of the blood through the cuvette was pulsatory and synchronous with the pulse. This phenomenon, probably related to the introduction of a rigid chamber into the otherwise distensible artery, did not interfere with spectrophotometry. In the extension of this technique automatic recording would be profitable. (4) Owing to the presence of pontamine fast pink and lack of exact information concerning its level of maintenance in the blood stream, some uncertainty exists in the extrapolation of the true spectra from the data obtained in the turbid state (19, 20). However, reliance may be placed upon a direct comparison of the extinction data obtained under varying conditions, and the agreement of results with those found on hemolyzed blood. The base-line of comparison was afforded by assuming that the spectrum yielded when the animal was under 100 per cent oxygen represented complete saturation (100 per cent HbO_2). A simplified extrapolation procedure (20) was used.

TABLE I

Agreement in Total Pigment Concentration ("Total Hemoglobin"), Measured Spectrophotometrically by Different Procedures

In all cases the values are in mM per liter (referred to the equivalent weight of 16,700) in the original, undiluted blood. Concentration is calculated from ϵ (upon the sample measured) divided by appropriate, corresponding ϵ (the standard, or mean value, used as an absorption constant). The standard ϵ values employed were 11.50 for MHbCN at 540 mμ (same for diluted as undiluted), 15.13, 8.73, and 14.62 for HbO_2 (diluted 1:100) at 578, 562, and 542 mμ respectively, 15.08, 8.62, and 14.75 for HbO_2 (undiluted, whole blood) at 578, 562, and 542 mμ respectively, 13.45 for Hb at 555 mμ (same for diluted as undiluted), and 5.22 at 505 mμ, an isobestic point for HbO_2, Hb, and mixtures of the two.

Blood source	Treatment	Total pigment measured as			
		MHbCN	HbO_2*	Hb†	Isobestically
Dog 3	Diluted, 1 cm. cuvette	10.33	10.27	10.33	
	Undiluted,‡ 0.007 cm. cuvette		10.38	10.35	10.44, HbO_2* 10.33, Hb† 10.33, M§
" 3	Diluted, 1 cm cuvette	11.38	11.33	11.40	
	Undiluted,‡ 0.007 cm. cuvette		11.47	11.41	11.35, HbO_2* 11.47, Hb† 11.35, M‖
" 4	Diluted, 1 cm cuvette	9.78	9.74	9.78	
	Undiluted,‡ 0.007 cm. cuvette		9.70	9.68	9.72, HbO_2* 9.72, Hb† 9.72, M‖

* Blood sample completely oxygenated.

† Blood sample deoxygenated by means of $Na_2S_2O_4$.

‡ Hemolyzed (saponized), oxalated whole blood.

§ The hemolyzed, arterial sample, unexposed to air, taken at end inspiration.

‖ The hemolyzed, arterial sample, unexposed to air, taken at end expiration.

The following pertinent data were secured. (1) In the circulating blood the concentration of pigment remained relatively constant (order of 5 per cent) over a period of several hours. (2) Intravenous injection of adrenalin (0.05 mg.) produced hemoconcentration of the order of 10 to 15 per cent, definitely observable within 4 minutes and maximal at 7 minutes after injection. (3) The oxygen saturation was maintained at a level of 97 to 98 per cent during the inhalation of room air. When this was changed

TABLE II

Direct Spectrophotometric Determination of Saturation of Hemoglobin in Arterial Blood

Species	Total pigment*	Σ_T†	Fraction Hb, $r = (\Sigma_p/\Sigma_T)$†	Fraction HbO_2, $1 - r$	Saturation, $(1 - r) \times 100$	Respiratory phase at sampling
	mM per l				*per cent*	
Dog 3‡	10.33	15.89	0.018	0.982	98.2	End inspiration
	11.47	15.89	0.013	0.987	98.7	" "
	11.35	15.89	0.045	0.955	95.5	" expiration
" 4‡	9.72	15.89	0.017	0.983	98.3	" inspiration
	9.78	15.89	0.034	0.966	96.6	" expiration
" 5	10.54	15.89	0.015	0.985	98.5	Undetermined
" 6	9.87	15.89	0.013	0.987	98.7	"
Average........					98.5§	
Man, W. K.	8.03	16.41‖	0.019	0.981	98.1	End expiration
	8.19	16.37‖	0.020	0.980	98.0	" "
" H. R.	8.63	16.39‖	0.015	0.985	98.5	" "
	8.55	16.34‖	0.017	0.983	98.3	" "
" H. G. S.¶	8.57	16.41‖	0.007	0.993	99.3	" inspiration
	8.14	16.46‖	0.009	0.991	99.1	" expiration
" R. C. L.**	7.76	16.36‖	0.010	0.990	99.0	" "
" L. J. D.††	9.12	16.32‖	0.018	0.982	98.2	" "
Average........					98.6	

* Total pigment determined as MHbCN. To obtain gm. per 100 ml., multiply the values by factor 1.67.

† For symbols see the text under "Methods."

‡ Nembutal anesthesia.

§ Values at end expiration omitted from the average.

‖ Mean Σ_T in man = 16.38; standard deviation = 0.04 ± 0.01.

¶ Oxygen tension (pO_2), determined by Dr. J. H. Comroe, Jr., ((4); unpublished work of Comroe and Dripps) upon an arterial sample withdrawn within several minutes of the sample used for spectrophotometry, yielded the value 98.5 mm. of Hg.

** A controlled diabetic, taking insulin.

†† A mild smoker, who abstained from smoking for 3 days prior to femoral puncture.

to 10 per cent oxygen, the percentage saturation promptly fell to 75 to 80. (4) Samples trapped (see "Methods") at end expiration consistently showed a lower saturation (95 to 96 per cent) than corresponding samples (trapped several minutes later) at end inspiration (98 per cent saturation) in these nembutalized animals breathing ten to twelve times per minute.

Observations on Hemolyzed (Oxalated, Saponized) Arterial Blood—The data recorded in Table I upon the agreement of total pigment concentration, measured as HbO_2 (after oxygenation), Hb (after deoxygenation with $Na_2S_2O_4$), MHbCN (after conversion with ferricyanide and cyanide), and isobestically at 505 mμ indicate that the spectrophotometric technique does not reveal the presence in measurable amount of MHb (besides HbO_2 and Hb) in freshly drawn dog arterial blood, determined promptly. As an added check in several instances (both in dog and human) KCN alone was added to the freshly drawn arterial blood, exposed to air. No change in spectrum could be demonstrated.

The data on the direct spectrophotometric determination of arterial saturation are collected in Table II. The values are uniformly high (98.0 to 99.3 per cent), and average 98.5 per cent in the dog and 98.6 per cent in man. The influence of respiratory phase, observed in the circulating blood, is confirmed by the measurements upon the hemolyzed samples from anesthetized dogs. This effect, however, was not demonstrable in unanesthetized man.

DISCUSSION

If the present results are correct, the conclusion must be drawn that the values for hemoglobin saturation in arterial blood at sea level have been underestimated by the indirect gasometric procedure. The causes for error probably reside, not in the manometric technique *per se*, but in the nature of the blood and its manipulation. This is in accord with the recent study of sources of error in the estimation of saturation by Roughton, Darling, and Root (3),[2] who conclude that hemoglobin saturation is underestimated by about 2 per cent in the gasometric method. According to them, the average value for hemoglobin saturation in arterial blood should be raised from 95 to 97 per cent. Dr. Boothby kindly has permitted us to quote corroborative evidence obtained by him and Dr. Robinson in man with the oximeter (25). It was customary to set the oximeter to read 95 per cent saturation when the subjects breathed room air. Boothby and Robinson, however, found that when the oximeters are set to read 100 per cent with the subjects breathing oxygen the saturation upon change to breathing room air is consistently 96 to 98 per cent.

Since percentage saturation has been employed in estimating oxygen tension from the dissociation curve, it is of interest that our values of the order of 98.0 and 98.5 per cent saturation correspond on a typical curve for man (8) to pO_2 values of 97 mm. and 100 mm. of Hg respectively. In one of the men (H. G. S., Table II), whose arterial saturation was 99.1 per cent, an independent determination of pO_2 by Dr. Comroe (4) yielded a value of 98.5 mm. With an improved technique Comroe and Dripps (unpublished work) have recently obtained an average value of 97.1 mm. for arterial pO_2. The independent analytical procedures therefore are in close accord. The ΔpO_2 between alveolar air and arterial blood would appear to be virtually erased whether the pO_2 is deduced from our values for saturation or obtained by direct determination.

The effect of phase of respiration on arterial oxygen saturation, demonstrated in the anesthetized dogs, confirms Kramer and Sarre's finding (23), also in the anesthetized animal. The same factor may be operative in unanesthetized man, but of a magnitude too small to be measured. Further observations are contemplated.

It may be proper to raise the question whether pure HbO_2 has ever been available for study. Perhaps the pigment in preparations from completely oxygenated blood is always contaminated with a minimal amount of MHb. When solutions prepared from fresh, fully oxygenated normal blood are treated with cyanide, the spectrophotometric technique fails to disclose changes in the direction suggesting the formation of MHbCN. On this basis the conclusion may be drawn that if preformed MHb is present its concentration must be of the order of 0.5 per cent of the total pigment, or lower, an amount beyond the possibilities of differentiation by present techniques. Roughton and his colleagues (3)[2] have confirmed Ammundsen (13) that after treatment with dithionite ($Na_2S_2O_4$) there is an average increase in CO capacity of the order of 3 per cent. This is a perplexing problem. The 3 per cent cannot represent MHb alone, since 1.0 to 1.5 per cent should be readily detectable by the spectrophotometric methods used in the present study (17). Other substances besides MHb have CO capacity after reduction, *e.g.*, ferrohemin and nitrogenous ferroporphyrins (28). It is unfortunate in the evaluation of this phenomenon that parallel determinations cannot be made of O_2 capacity after $Na_2S_2O_4$, owing to the formation of oxidants from the latter. The possibility remains that part of the effect on CO capacity may be an artifact. It would also be desirable to accumulate sufficient data for a comparison of the behavior of arterial and venous blood in this connection. Most of the analyses have been performed upon venous blood, although Roughton *et al.* (3)[2] state that this makes little difference.

The factors which influence the equilibria $HbO_2 \rightleftharpoons Hb \rightleftharpoons MHb$ in blood are indeed complicated. MHb may disappear from blood after it is drawn (14). On the other hand, MHb may be formed in blood and blood solutions on standing (17, 27). Roughton and associates (3)[2] found an increase in O_2 capacity (without $Na_2S_2O_4$) in blood standing at room temperature. In the best experiments this effect amounted to 1.1 per cent in terms of total pigment, and was interpreted to represent the quantity of the total MHb which had reverted to HbO_2 (through Hb). Whereas many hemoglobin derivatives combine with CO, very few (only Hb, under normal pH conditions in blood) can unite with O_2. We believe that the 1.1 per cent may approach the value for total MHb in Roughton's samples, mainly of venous blood.

We are indebted to Miss H. Lorraine Leidy and Dr. H. H. Pennes (Department of Pharmacology) for technical assistance, and particularly to Dr. H. D. Bruner (Harrison Department of Surgical Research) for performing the femoral arterial punctures in man.

SUMMARY

The Drabkin and Austin special cuvette of 0.007 cm. depth (18) has been ulitized to extend the spectrophotometric technique to the study of blood *in vivo* and to the determination of arterial saturation.

1. The continuous spectrophotometric observation of circulating blood has been successfully performed in nembutalized dogs.
2. The hemoglobin saturation of hemolyzed arterial blood has been determined in dog and man by direct spectrophotometry. The analyses were performed within 2 to 3 minutes after the blood was withdrawn from the subject. The saturation was uniformly high in both species. It varied from 98.0 to 99.3 per cent, and had average values of 98.5 and 98.6 per cent in the dog and man respectively.
3. The phase of the respiratory cycle was found to be a factor in the value of arterial saturation in the anesthetized dogs, but not in unanesthetized man.
4. The significance and interpretation of the findings have been discussed.

BIBLIOGRAPHY

1. Drabkin, D. L., and Schmidt, C. F., *Am. J. Med. Sc.*, **208**, 133 (1944).
2. Drabkin, D. L., Schmidt, C. F., Bruner, H. D., and Pennes, H. H., *Am. J. Med. Sc.*, **208**, 135 (1944).
3. Roughton, F. J. W., Darling, R. C., and Root, W. S., *Am. J. Med. Sc.*, **208**, 132 (1944).
4. Comroe, J. H., Jr., *Am. J. Med. Sc.*, **208**, 135 (1944).
5. Lennox, W. G., and Gibbs, E. L., *J. Clin. Invest.*, **11**, 1155 (1932).
6. Keys, A., and Snell, A. M., *J. Clin. Invest.*, **17**, 59 (1938).
7. Cullen, S. C., and Cook, E. V., *Am. J. Physiol.*, **137**, 238 (1942).
8. Bock, A. V., Field, H., Jr., and Adair, G. S., *J. Biol. Chem.*, **59**, 353 (1924).

9. Bock, A. V., Dill, D. B., Edwards, H. T., Henderson, L. J., and Talbott, J. H., *J. Physiol.*, **68,** 277 (1929–30).
10. Van Slyke, D. D., and Neill, J. M., *J. Biol. Chem.*, **61,** 523 (1924). Van Slyke, D. D., *J. Biol. Chem.*, **73,** 121 (1927).
11. Ammundsen, E., and Trier, M., *Acta med. Scand.*, **101,** 451 (1939).
12. Taylor, D. S., and Coryell, C. D., *J. Am. Chem. Soc.*, **60,** 1177 (1938).
13. Ammundsen, E., *J. Biol. Chem.*, **138,** 563 (1941).
14. Wendel, W. B., *J. Clin. Invest.*, **18,** 179 (1939). Cox, W. W , and Wendel, W. B., *J. Biol. Chem.*, **143,** 331 (1942). Heubner, W., and Stuhlmann, M., *Arch. exp. Path. u. Pharmakol.*, **199,** 1 (1942).
15. Kiese, M., *Biochem. Z.*, **316,** 264 (1944).
16. Drabkin, D. L., in Glasser, O., Medical physics, Chicago, 967 (1944).
17. Austin, J. H., and Drabkin, D. L., *J. Biol. Chem.*, **112,** 67 (1935–36).
18. Drabkin, D. L., and Austin, J. H., *J. Biol. Chem.*, **112,** 105 (1935–36).
19. Drabkin, D. L., and Singer, R. B., *J. Biol. Chem.*, **129,** 739 (1939).
20. Drabkin, D. L., *Proc. Am. Soc. Biol. Chem* , *J. Biol. Chem.*, **140,** p. xxxiv (1941).
21. Lowry, O. H., Smith, C. A., and Cohen, D. L., *J. Biol. Chem.*, **146,** 519 (1942).
22. Kramer, K , *Z. Biol* , **96,** 61 (1935).
23. Kramer, K , and Sarre, H., *Z. Biol.*, **96,** 76, 89, 101 (1935).
24. Matthes, K., and Gross, F., *Arch. exp. Path. u. Pharmakol.*, **191,** 369, 381, 391, 523, 706 (1939).
25. Millikan, G. A., *Rev. Scient Instruments*, **13,** 434 (1942).
26. Quick, A. J., The hemorrhagic diseases and the physiology of hemostasis, Springfield and Baltimore, 98, 106 (1942).
27. Drabkin, D. L., and Austin, J. H., *J. Biol. Chem.*, **112,** 51 (1935–36).
28. Drabkin, D. L , *J. Biol Chem* , **146,** 605 (1942).

TRANSAMINATION IN BACTERIA

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The enzyme-catalyzed transamination reaction involves the intermolecular transfer of an amino group from an α-amino acid to an α-keto acid. Since the discovery of this reaction by Braunstein and Kritzmann (1) in muscle tissue, the reaction has been studied in some detail, particularly in animal tissues (2, 3). More recently (4), a study of this reaction in germinating oat seedlings revealed the presence of a highly active transaminating system which was interpreted to play a direct rôle in protein synthesis. In contrast to the studies on plant and animal tissues, reports on the presence or absence of this reaction in bacteria have been equivocal. Thus, Adler *et al.* (5) stated that *Bacillus coli* catalyzed transamination but no experimental data were presented in support of this. Diczfalusy (6), working with *B. coli* and other organisms, reported negative results in a more elaborate study. On the other hand, Cohen (2) referred to unpublished data which showed a low but definite transaminase activity in *B. coli*. In view of the fact that bacteria are rapidly growing organisms, and thus must be capable of extremely rapid protein synthesis, it seemed important to study the transamination reaction in a variety of organisms. In this paper, experiments are reported which establish the presence in several species of bacteria of a potent transaminase system which catalyzes the following reaction.

(1) $l(+)$-Glutamic acid + oxalacetic acid → α-ketoglutaric acid + $l(-)$-aspartic acid

Procedure

Preparation of Bacterial Suspensions—In general the desired organisms were cultivated on tryptose agar contained in Kolle flasks for 18 to 24 hours at 37°. For the streptococci and the pneumococcus, 0.1 per cent glucose tryptose agar was employed, while *Bacillus welchii* was cultivated anaerobically on the same medium in a Novy jar. *Azotobacter vinelandii* was grown in Burke's synthetic medium at room temperature under aerobic conditions. After incubation the organisms were harvested, suspended in physiological salt solution, centrifuged, washed twice, and finally resuspended in M/15 phosphate buffer solution of the desired pH. Total nitrogen content was determined on all suspensions by a micro-Kjeldahl method.

Preparation of Substrates and Incubation Mixtures—Glutamic and oxalacetic acids were added as neutral salts in concentrations of 0.12 M and 0.2 M respectively to give a final concentration of each of 0.021 M. The bacterial suspension and buffer solution were added to the main compartment of the Warburg cup, while the glutamic and oxalacetic acids were placed in the side arm in 0.5 ml. and 0.3 ml. amounts respectively. Inasmuch as it was quickly ascertained that transaminase activity could not be detected under aerobic conditions, all incubations were carried out anaerobically. Anaerobiosis was maintained by passing tank nitrogen through the cups. After 10 minutes shaking at 38° in a water bath, the cups were tipped, the reaction allowed to take place for the desired period of time, and then stopped by the addition of 1 ml. of 10 per cent sulfuric acid. The contents of the cups were washed into graduated centrifuge tubes, 1 ml. of 10 per cent sodium tungstate added, and the volumes read. The mixtures were then filtered, the volumes again recorded, and the solutions boiled for about 45 minutes to destroy any remaining oxalacetic acid. After adjusting to the original volumes with distilled water, an aliquot was taken for aspartic acid determination according to the method previously described (7). All determinations were carried out in at least triplicate. Rates of transamination are expressed as Q_{TN} values.

$$Q_{TN} = \frac{\text{microliters aspartic acid formed}}{\text{mg. N} \times \text{hrs.}}$$

Results

Preliminary experiments were performed with suspensions of *Bacillus coli* with incubation periods of 1 and 2 hours, on the assumption that the previously reported negative results with bacteria may have been due to a very low transaminase activity. The results of these experiments indicated definite transaminase activity but of a low magnitude, *i.e.*, 5 to 10 per cent transamination with Q_{TN} values of 40 to 80. These values were in the same range as those previously found by Cohen (2). Further experiments, however, showed that *Bacillus coli* was able to deaminize and decarboxylate aspartic acid at such a rapid rate anaerobically that one could not expect any significant accumulation of this end-product. Thus it was found that of 1344 microliters of added aspartic acid, 45 per cent was destroyed in 5 minutes, and 75 per cent in 15 minutes under conditions simulating those employed in studying transamination. This effect is demonstrated in Fig. 1, in which it is seen that the apparent rate of transamination decreases with time. As a matter of fact, in the light of the rapid rate of aspartic acid disappearance, it can be seen from Fig. 1 that transamination is actually a very rapid reaction, and that the low rates observed after 30 or more minutes incubation are due to the rapid decarboxylation and deamination of aspartic acid. That the Q_{TN} values at even 5 minutes are not maximum is shown by experiments in which 1 minute incubation times were used. Q_{TN} values as high as 3900 were obtained under these conditions. However, because of practical considerations, a 5 minute incubation time was employed in all the experiments reported.

Dilution experiments with increasing incubation times revealed that the same relative activity persisted in the system or systems responsible for deamination and decarboxylation, and consequently more dilute bacterial suspensions with longer incubation periods could not be employed to study the kinetics of this system.

Effect of Bacterial Concentration—The results shown in Fig. 2 indicate that transaminase activity in *Bacillus coli* is directly proportional to the concentration of bacteria within the limits of 1.95 to 7.8 mg. of bacterial nitrogen. In this range the Q_{TN} values were quite constant with an average of 700. No appreciable transaminase activity was observed with a bacterial nitrogen content of 0.98 mg.

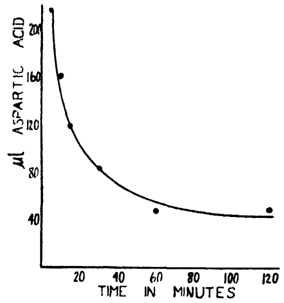

FIG. 1

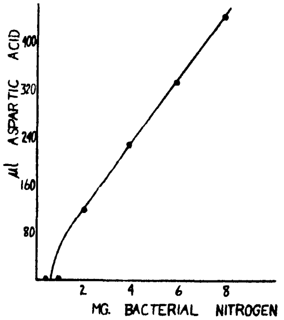

FIG. 2

FIG. 1. Effect of incubation time on transamination with *Bacillus coli*; 3.20 mg. of bacterial nitrogen per flask; pH 7.6; abscissa, length of incubation time in minutes; ordinate, microliters of aspartic acid formed.

FIG. 2. Effect of bacterial nitrogen concentration on transamination with *Bacillus coli*; pH 7.6; incubation time, 5 minutes.

pH Optimum—It is seen from Fig. 3 that the pH optimum for the *Bacillus coli* system is about 8.5. This is essentially the same as that found for oat seedlings (4), but is higher than that reported for purified animal transaminase preparations (8). The latter had a pH optimum of 7.5. Previous to the determination of the pH optimum, all experiments were carried out at pH 7.6. Subsequent to this all experiments were run at pH 8. The optimal pH was not employed, since it seemed desirable to use a phosphate buffer solution.

Effect of Temperature—The optimal temperature for the *Bacillus coli* transaminase system lies close to 32° (Fig. 4). It is somewhat surprising to find that the rate of transamination decreases rapidly above this temperature. On the other hand, since intact cells were employed, it is quite consistent with the optimal growth temperature for *Bacillus coli*, which is in the neighborhood of 32°. As can be seen from Fig. 4, between 10–32° the rate of transamination is approximately doubled with each 10° rise in temperature.

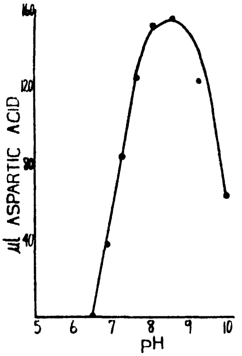

FIG. 3

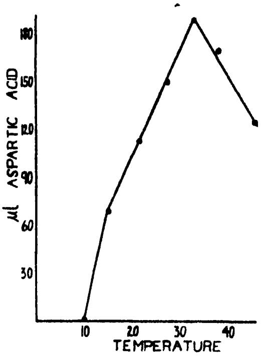

FIG. 4

FIG. 3. Effect of pH on transamination with *Bacillus coli*; 2.63 mg. of bacterial nitrogen per flask; incubation time, 5 minutes.

FIG. 4. Effect of temperature on transamination with *Bacillus coli*; 2 76 mg. of bacterial nitrogen per flask; pH 8 0; incubation time, 5 minutes.

TABLE I

Q_{TN} Values for Representative Species of Bacteria (5 Minutes Incubation)

18 to 24 hour cultures were employed; pH 8.0.

Organism	Q_{TN}
Bacillus coli	890
" *dysenteriae* (Shiga)	685
" *typhosus*	1135
" *proteus*	1610
" *pyocyaneus*	800
Azotobacter vinelandii	1575
Staphylococcus aureus	910
" *albus*	950
Bacillus welchii	1170
Streptococcus hemolyticus	865
" *viridans*	900
Pneumococcus Type I	845

Q_{TN} Values in Different Species of Bacteria—It appeared of interest to assay quantitatively different bacterial species for transaminase activity. All incubations were carried out for 5 minutes at pH 8 and 38°. While these conditions were as close to optimal as could be obtained practically for *Bacillus coli*, it is not at all certain that these conditions are optimal for the other bacteria investigated. Consequently, one must assume that the values reported in Table I are minimal. It is suggested from the data that different organisms within a species tend to have a transaminase activity of the same order of magnitude. This is particularly suggestive in the case of the staphylococci and the streptococci. *Bacillus proteus* and *Azotobacter vinelandii* show the greatest transaminase activity of the organisms studied. Since the latter is a nitrogen-fixing organism with high metabolic activity, the presence of a highly active transaminase system is of considerable interest.

DISCUSSION

The data presented in this paper provide unequivocal evidence for the occurrence of a highly active transaminase system in several species of bacteria. Diczfalusy's (6) failure to demonstrate transaminase activity in *Bacillus coli* with the following reactions

(2) $l(-)$-Aspartic acid + α-ketoglutaric acid → $l(+)$-glutamic acid + oxalacetic acid

(3) α-Ketoglutaric acid + $l(+)$-alanine → $l(+)$-glutamic acid + pyruvic acid

is understandable, since both these reactions proceed very slowly as compared with Reaction 1 (9). As a matter of fact the rates of Reactions 2 and 3 would proceed so slowly that the end-products measured by Diczfalusy, *viz.*, pyruvic and oxalacetic acids, would be destroyed by side reactions before they could accumulate in measurable quantities. Both these substances are rapidly utilized anaerobically by *Bacillus coli* and many other organisms. In the case of Reaction 2, which proceeds faster than Reaction 3 (9), the rapid deamination and decarboxylation of aspartic acid, demonstrated in the present paper, would preclude the measurement of any appreciable transamination under the conditions employed by Diczfalusy. On the other hand, glutamic acid is relatively inert, and had this end-product been measured, a small amount of transaminase activity might have been demonstrated at least for Reaction 2.

It is a matter of some interest to compare the relative rates of transamination in bacteria, higher plants, and animal tissues. In Table II some selected previously published values, converted to Q_{TN} values, for plant and animal tissues are presented. It is apparent that the highest Q_{TN} value obtained for *Bacillus coli* exceeds those found with animal tissues. Oat seedlings give the highest value of any unpurified preparation studied to date, showing roughly one-half the activity of the purified heart muscle preparation. On the basis of the experiments with *Bacillus coli* it is more than likely that the values presented in Table I are 3 to 5 times lower than the optimal Q_{TN} values. It can therefore be seen that most bacteria exceed animal tissues in their transaminase activity, and in some organisms such as *Azotobacter vinelandii* and *Bacillus proteus* the Q_{TN} values obtained under optimal conditions would probably be several fold greater than those reported for animal tissues. Thus, in contrast to previous impressions, transamination is a more rapid reaction in bacteria and higher plants than in animal tissues. In view of the more rapid nitrogen metabolism and protein synthesis in these lower forms, such a relationship is quite understandable.

TABLE II

Q_{TN} Values of Bacillus Coli, Animal, and Plant Tissues

Tissue	Q_{TN}	Bibliographic reference No
Bacillus coli	3,900	Present paper
Oat seedlings (96 hrs.)	5,650	4
Brain (rat)	2,800	9
Liver "	2,200	9
Kidney "	1,750	9
Heart muscle (rat)	3,330	9
Purified transaminase (beef heart muscle)	10,300	8

The data reported here are consistent with the view that transamination is in some manner directly concerned with the synthesis of protein (4). The Q_{TN} values for the bacteria reported in this paper are considerably higher than any other metabolic quotients reported for these organisms. At least in the case of *Bacillus coli*, a search of the literature fails to reveal any metabolic Q values on a nitrogen basis which approach the Q_{TN} values given in this paper.

SUMMARY

1. The transamination reaction

$l(+)$-Glutamic acid + oxalacetic acid → α-ketoglutaric acid + $l(-)$-aspartic acid

has been studied in several bacterial species. The optimal pH for this reaction in *Bacillus coli* lies at about 8.5, and the optimal temperature appears to be 32°.

2. Q_{TN} values for twelve organisms are presented, all of which are of a high order of magnitude. Optimal Q_{TN} values for *Bacillus coli* exceed those reported for animal tissues.

BIBLIOGRAPHY

1. Braunstein, A. E., and Kritzmann, M. G., *Enzymologia*, **2**, 129 (1937).
2. Cohen, P. P., *Federation Proc.*, **1**, 273 (1942).
3. Cohen, P. P., in A symposium on respiratory enzymes, Madison, 210 (1942).
4. Albaum, H. G., and Cohen, P. P., *J. Biol. Chem.*, **149**, 19 (1943).
5. Adler, E., Hellström, V., Günther, G., and von Euler, H., *Z. physiol. Chem.*, **255**, 14 (1938).
6. Diczfalusy, C., *Biochem. Z.*, **313**, 75 (1942).
7. Cohen, P. P., *J. Biol. Chem.*, **136**, 565 (1940).
8. Cohen, P. P., *J. Biol. Chem.*, **136**, 585 (1940).
9. Cohen, P. P., and Hekhuis, G. L , *J. Biol. Chem.*, **140**, 711 (1941).

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CONCENTRATION OF α-AMINO ACID NITROGEN IN PLASMA OF NORMAL SUBJECTS*

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(Received for publication, October 3, 1944)

The recent development of the ninhydrin-carbon dioxide method by Van Slyke and associates (1–4), the use of their technique in studying protein metabolism in nephrosis (5–8), and the reports of intravenous protein digest administration in man (9) have stimulated interest anew in the quantitative determination of the circulating amino acids. Previously published normal values obtained by the ninhydrin-carbon dioxide method (1, 10) have not conclusively defined the range of α-amino acid nitrogen in the plasma of normal subjects in the postabsorptive state. Additional normal values are presented because only a few of the subjects of Hamilton and Van Slyke (1) were known to be in the postabsorptive state, and because the lower boundary of the normal range in Cramer and Winnick's (10) data was below that assumed by Farr (8). In this paper are presented values on the circulating α-amino acid nitrogen in the plasma of thirty-seven normal individuals, sixteen females and twenty-one males, in the postabsorptive state.

Method

The ninhydrin-carbon dioxide method of Hamilton and Van Slyke (1) was used. Blood was drawn from the antecubital vein in a dry syringe and placed in a centrifuge tube containing 0.66 mg. of crystalline heparin. Blood was centrifuged and the plasma separated from the cells within 1 hour of the time the blood was obtained. Hemolysis was never observed. Occasionally plasma was allowed to stand for about an hour before addition of the picric acid solution. After the addition of picric acid solution, the technique was conducted as rapidly as possible through heating in a boiling water bath for 20 minutes. Subsequent to this procedure the determination of carbon dioxide was performed whenever convenient during the next 36 hours.

The all-glass reaction vessel was used throughout. Dr. Hamilton[1] advised the use of a second drop of caprylic or octyl alcohol when the ninhydrin was added. Also he called to our attention that the cooling in ice water after preboiling should not be prolonged more than 1½ minutes. Longer cooling in ice water may result in greater absorption of atmospheric carbon dioxide than would occur at room temperature.

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* This work was aided in part by grants from the Fluid Research Fund of Yale University School of Medicine.

† Most of the data in this paper are taken from the thesis presented by Calvin W. Woodruff in partial fulfilment of the requirements for the degree of Doctor of Medicine at Yale University School of Medicine, 1944.

[1] Hamilton, P. B., personal communication.

Carbon dioxide from twice recrystallized *dl*-alanine was determined theoretically in four experiments. The average of the calculated quantity of *dl*-alanine was 98.6 per cent of the amount added, with an average deviation from the mean of 0.3 per cent. In four other experiments *dl*-alanine was added to the picric acid filtrate of plasma. The average calculated quantity of *dl*-alanine was 100.2 per cent with an average deviation from the mean of 2.2 per cent. In the determinations on plasma to be presented in this paper, the average difference between aliquots was 0.06 mg. of α-amino acid nitrogen per 100 cc. or 1.3 per cent. The greatest difference was 0.16 mg. per 100 cc. and the majority of the determinations had a difference of less than 0.10 mg. per 100 cc. or 2.4 per cent. This corresponds to the error found in the theoretical analyses for *dl*-alanine.

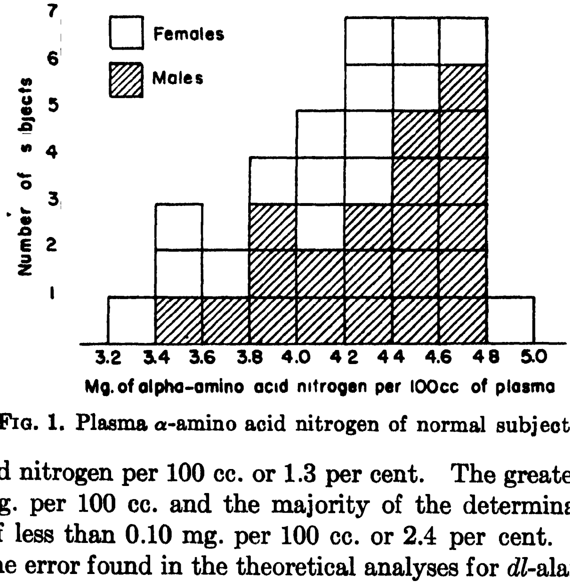

FIG. 1. Plasma α-amino acid nitrogen of normal subjects

The results are reported uncorrected for urea. A normal non-protein nitrogen is assumed. All the values are the averages of duplicate analyses. They are expressed as mg. of α-amino acid nitrogen per 100 cc. of plasma.

In Fig. 1 are presented the values of the α-amino acid nitrogen in the plasma of thirty-seven normal subjects in the postabsorptive state. Of these medical students, nurses, and laboratory workers, sixteen were females and twenty-one males. They were all in good health and, with a few exceptions, between the ages of twenty and thirty years. The values ranged from 3.37 to 4.97, with an average of 4.23 mg. of α-amino acid nitrogen per 100 cc. of plasma. All except two values fell between 3.59 and 4.76 mg. per cent. In Fig. 1 the curve of frequency distribution may be skewed to the right, instead of following the symmetrical normal frequency distribution curve, but a larger number of normal values would be required to confirm this. The average value for the sixteen females was 4.13 and for the twenty-one males 4.31 mg. of α-amino acid per 100 cc. of plasma.

DISCUSSION

In this series of thirty-seven subjects in the postabsorptive state, the average normal value of 4.23 mg. of α-amino acid nitrogen per 100 cc. plasma is essentially the same as 4.07 mg. per 100 cc. reported by Hamilton and Van Slyke (1) and as 4.2 reported by Cramer and Winnick (10). Due to their use of a correction for plasma urea concentration, Hamilton and Van Slyke's values should be about 0.1 mg. per cent lower than those of Cramer and Winnick and of our subjects. The general results on the normal average level show basic agreement among all who have used this method. The range of values on our thirty-seven normal subjects, from 3.37 to 4.97 mg. per cent of α-amino acid nitrogen, is almost identical with that of Hamilton and Van Slyke, from 3.35 to 4.72 mg. per cent.

Only three of the thirteen values of Hamilton and Van Slyke from plasma of ten different subjects were made when the subjects were known to be in a postabsorptive state. In two of their subjects, α-amino acids in the plasma were determined, both in the fasting and non-fasting states. In each instance the fasting value was lower: 3.35 *versus* 4.45, and 4.32 *versus* 5.00 mg. per cent of α-amino acid nitrogen. Since protein ingestion is known to elevate the α-amino acid nitrogen in the plasma (11, 12), it was impossible to evaluate the effect on the plasma amino acids of the ingestion of food by their other normal subjects. In fact, in our own experiments on two subjects the ingestion of 42 gm. of protein, chiefly in the form of hamburger steak, resulted in a rise of about 1.0 mg. per cent of α-amino acid nitrogen in the plasma. The α-amino acid nitrogen in the plasma rose from 3.77 to 4.87 mg. per cent in one subject and from 3.63 to 4.64 mg. per cent in the other. In spite of the fact that Hamilton and Van Slyke's subjects were possibly not in the postabsorptive state, their minimum, maximum, and average values were in close agreement with those in this series.

The data of Cramer and Winnick (10), obtained on twenty fasting convalescent hospital patients, show a much greater range of values. Their average difference between duplicates (3.3 per cent) was greater than that reported by Hamilton and Van Slyke (1). Their subjects were convalescent patients and perhaps should not be considered normal for that reason. The range of from 2.3 to 7.3 mg. of α-amino acid nitrogen per 100 cc. which they report includes one value (2.3 mg. per 100 cc.) which is below the critical level of 2.50 mg. per 100 cc. established by Farr in nephrosis (8), and must be considered abnormal. Since no values in normal individuals above 5.00 mg. per 100 cc. have been reported, the higher values from Cramer and Winnick's convalescent patients cannot be properly evaluated. The highest non-fasting value which has ever been obtained in our laboratory, 5.82 mg. of α-amino acid nitrogen per 100 cc. of plasma, was in a subject who neglected to omit breakfast before coming to the laboratory.

Many adaptations of various methods have been used in studying values of amino nitrogen in normal subjects in the postabsorptive state. Workers with Folin's colorimetric method (13), or some adaptation thereof, have found that normal fasting values for whole blood fell between 5.0 and 8.0 mg. of amino nitrogen per 100 cc. in the great majority of the individuals studied (14, 15). With the nitrous acid method the range given by Bock (16) of 6.0 to 8.0 mg. of amino nitrogen per 100 cc. of whole blood and an average of 4.8 mg. per 100 cc. of plasma have been generally accepted. Among the workers who use the different gasometric methods, attention has been directed mainly to the plasma. MacFadyen (3) found higher values in serum than in plasma and considered plasma from heparinized blood would yield a more correct indication of the circulating amino acids. With the nitrous acid method values between 3.75 and 6.50 mg. of amino nitrogen per 100 cc. of plasma have been reported (1, 17). This range is in general agreement with determinations by the ninhydrin-carbon dioxide method, except that, as pointed out by Hamilton and Van Slyke (1), the nitrous acid method usually has given slightly higher values because some glutamine amide nitrogen is included in the determination.

No previous studies define the day to day variation of the α-amino acid nitrogen in the plasma in any one individual. The differences in the values of Hamilton and Van Slyke's two subjects in both the postabsorptive and absorptive states were 1.10 and 0.68 mg. per cent. This is a greater variation than was observed in one subject of this series. On four different occasions throughout a period of 1 month the α-amino acid nitrogen in the plasma was determined in one male in the postabsorptive state. The values were 3.73, 3.38, 3.56, and 3.77 mg. per cent of α-amino acid nitrogen in the plasma and the minimum and maximum differed by 0.39 mg. per cent. The difference as compared with the greatest value was 9.6 per cent. This difference is more than 4 times the experimental error and is significant, although the extent of this variation is well within the normal range. What this variation means and whether or not it reflects the previous dietary history are questions remaining to be answered.

The absence of any significant difference in plasma amino acids between the sexes is in accord with previously published data (16, 18).

SUMMARY

By using the ninhydrin-carbon dioxide method of Hamilton and Van Slyke, α-amino acid nitrogen determinations were made on the plasma of sixteen female and twenty-one male normal human subjects in the post-absorptive state.

The values ranged from 3.37 to 4.97 with an average of 4.23 mg. of α-amino acid nitrogen per 100 cc. of plasma. No correction was made for plasma urea concentration.

No significant difference between males and females was found in the concentration of the α-amino acid nitrogen in the plasma.

BIBLIOGRAPHY

1. Hamilton, P. B., and Van Slyke, D D., *J Biol. Chem.*, **150**, 231 (1943).
2. MacFadyen, D. A , *J Biol Chem* , **145**, 387 (1942).
3. Van Slyke, D. D , Dillon, R T., MacFadyen, D. A., and Hamilton, P. B , *J. Biol. Chem* , **141**, 627 (1941).
4. Van Slyke, D D , MacFadyen, D. A., and Hamilton, P., *J. Biol. Chem.*, **141**, 671 (1941).
5. Emerson, K , Jr., and Van Slyke, D. D , *J Mt. Sinai Hosp* , **8**, 495 (1942).
6. Farr, L. E , *J. Pediat* , **16**, 679 (1940).
7 Farr, L E , *J Pediat* , **17**, 734 (1940).
8. Farr, L E , and MacFadyen, D A., *Am. J Dis Child* , **59**, 782 (1940).
9. Elman, R , and Weiner, D O., *J Am. Med Assn.*, **112**, 796 (1939).
10. Cramer, F B., Jr., and Winnick, T , *J Biol Chem.*, **150**, 259 (1943).
11. Barber, H. W., and Oriel, G H., *Lancet*, **2**, 1009 (1928).
12 Witts, L , *Quart J Med* , **22**, 477 (1929).
13. Folin, O , *J. Biol. Chem.*, **51**, 377 (1922).
14. Becher, E , and Herrmann, E., *Deut Arch klin Med.*, **171**, 547 (1931).
15. Greene, C. H , Sandiford, K , and Ross, H , *J. Biol Chem* , **58**, 845 (1924).
16. Bock, J. C., *J. Biol. Chem.*, **29**, 191 (1917).
17. Kirk, E., *J. Clin. Invest* , **12**, 1091 (1933)
18. Costantino, A., *Biochem Z.*, **51**, 91 (1913).

STUDIES IN MINERAL METABOLISM WITH THE AID OF ARTIFICIAL RADIOACTIVE ISOTOPES

VIII. TRACER EXPERIMENTS WITH RADIOACTIVE CALCIUM AND STRONTIUM ON THE MECHANISM OF VITAMIN D ACTION IN RACHITIC RATS*

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Study of the mode of action of vitamin D in rickets has led to varied conclusions concerning the mechanism whereby the mineralization of bone is promoted by this vitamin. The literature on the subject has been reviewed in another place by the writer (1).

A hypothesis that has met with much favor is that vitamin D increases the absorption of calcium from the gastrointestinal tract and the urinary excretion of phosphorus, but has no effect on either the fecal excretion or the absorption of phosphorus. Many investigators have felt that this hypothesis is inadequate and that, aside from its effects on absorption and excretion, vitamin D, in some manner, must also have a direct effect on the mineralization of bone.

From previous tracer experiments with radioactive phosphorus, Cohn and Greenberg (2) reached the conclusion that the vitamin acts to aid the conversion of organic to inorganic phosphorus in bone.

The work with radioactive phosphorus has now been followed up by an investigation of the mode of action of vitamin D in the healing of rickets by means of tracer experiments with radioactive calcium and strontium. The experimental results lead to the conclusion that vitamin D exerts its healing effect partly by favoring absorption of calcium from the intestinal tract and partly by exerting a direct influence on the process of mineralization in bone.

EXPERIMENTAL

The technique of the experiments was much the same as that previously used in the studies with radioactive phosphorus (2). Rachitic rats were obtained by rearing weaned young for 15 to 20 days on a modified Steenbock-Black rachitogenic ration (see (2)). Doses of 0.2 to 0.3 ml. of irradiated ergosterol (10,000 U. S. P. XI units of vitamin D per gm.) or cottonseed oil were administered orally to paired groups of rats 72 hours and 1 hour prior to administration of the radioactive materials.

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During the experimental period each rat was kept in a separate wire bottomed cage placed over a urine-feces separator. Collections of excreta were made at regular intervals. At the end of an experimental period, the rats were sacrificed and skinned. One femur was removed for ash analysis and the intestinal tract added to the last fecal collection. The rest of the carcass was placed in a beaker, covered with water, and then autoclaved for about 2 hours. This disintegrated the soft tissues and allowed the bones and teeth to be easily separated. Bones and incisor and molar teeth were collected, dried, weighed, and then ashed in an electrical muffle furnace. The disintegrated mass of soft tissues, with the skin added, was evaporated to dryness in an evaporating dish and also ashed. The ash was dissolved in HCl and made up to volume in volumetric flasks. Suitable aliquots were pipetted into ½ ounce lacquered ointment tins[1] and evaporated to dryness in preparation for measuring the radioactivity.

In a number of experiments such tissues and viscera as liver, heart, kidney, skin, and muscle were collected for separate measurement. This practice was discontinued, because the degree of accumulation of the labeled calcium and strontium in each of the above tissues was too small to be significant.

The radioactive isotopes Ca^{45} (one-half life, 180 days) and Sr^{89} (one-half life, 55 days) were prepared by bombarding the respective metals with deuterons in the University of California medical cyclotron (see (3)). Solutions of the lactate salts of the radioactive ions,[2] containing 0.5 to 2 mg. of Sr^* or 1.5 to 4 mg. of Ca^*, were administered by stomach tube or by intraperitoneal injection.

The radioactivity of each dose of Sr^* was approximately 10 microcuries and of each dose of Ca^* 0.02 microcurie (1 microcurie $= 2.2 \times 10^6$ counts on the Geiger-Müller counter).

The radioactivity of the Sr^* samples was measured with a Lauritsen electroscope (sensitivity, 4000 units per microcurie) and of the Ca^* samples on a scale of eight Geiger-Müller counter.[3] A bell type counter tube with a mica window (4) was used to estimate the soft β radiations emitted by the Ca^{45}. A correction for the amount of radiation self-absorbed was applied to the Ca^* samples. The correction was obtained from absorption curves, determined by adding increasing amounts of rat ash to samples of Ca^* lactate solutions of measured radioactivity. The correction for absorption was estimated from the weight of sample in each ointment tin.

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[1] Manufactured by the Buckeye Stamping Company, Columbus, Ohio.

[2] An atomic symbol with an asterisk represents the element labeled with a radioactive isotope.

[3] Built by the Cyclotron Specialities Company, Moraga, California.

The results of the experiments with Ca* were not as consistent as those with Sr*, probably because of errors in estimating self-absorption, and chance contaminations with Sr*.

The total of the radioactive Sr* or Ca* recovered from all samples of each animal was computed and the data corrected to a basis of 100 in each case. The range of the recovery varied from about 70 to 110 per cent of the activity of the dose given.

DISCUSSION

The average values of the experimental results are recorded in Tables I and II. The values of Ca* and Sr* are expressed in terms of the percentage of the administered dose. The data for Ca* and Sr*, while differing in degree, parallel each other and lead to the same conclusions. This agrees with previous studies, which have shown that in tracer doses the metabolism of strontium and calcium is closely akin (5, 6). Bone and tissues, however, show a lesser degree of accumulation of Sr* than of Ca*.

The partition of Sr* and Ca*, between excreta and the body, under the varying experimental conditions is shown in Table I. It will be observed that the excreta and the skeleton contain the bulk of the administered ions and that the amount present in the soft tissues is comparatively negligible. This is even more strikingly the case if one considers individual tissues such as skin, muscle, liver, kidney, etc. Because of the low degree of accumulation of Sr* and Ca* in the soft tissues, they were neglected in the consideration of the turnover of the above elements. No deductions of any significance were apparent from the data of Ca* and Sr* in the residual carcasses.

The data of Table I show that one of the actions of vitamin D is to promote the absorption of calcium from the intestinal tract. This point has been stressed in particular in recent times by Nicolaysen (7). The variation in the excretion of Ca* and Sr* under the different experimental conditions readily confirms this deduction.

Upon oral administration of a dose of Ca* or Sr* to a rachitic rat, the fraction excreted in the feces is very high, amounting to about 60 to 70 per cent in 3 days. Approximately 20 per cent is eliminated in the urine and the rest is retained in the body. In the vitamin D-treated animals there is a decrease in the quantity of Ca* and Sr* in the feces and an increase of the amount put out in the urine. Examination of the fecal contents of Ca* and Sr* of the injected rats shows little difference in the intestinal excretion between the rachitic and vitamin D-treated animals. This result indicates that the true intestinal excretion of calcium is approximately constant and is not appreciably affected by vitamin D. Using the fecal values of Ca* and Sr* of the injected rats as a rough measure of the intestinal excretion, one calculates that in the rachitic rats dosed with Ca* and Sr* orally about 40 per cent of the Ca* and 45 per cent of Sr* passed through the gastrointestinal tract unabsorbed, while in the vitamin D-treated animals only about 15 per cent of the Ca* and 30 per cent of the Sr* passed through unabsorbed.

The bones of the vitamin D-treated animals retain a larger proportion of the labeled metal ions than do those of the untreated rats, as would be expected. If the hypothesis, that the only action of vitamin D is to promote absorption of calcium from the digestive tract, were correct, the rachitic animals should retain as much of the injected Ca* and Sr* in the bone as do the vitamin D-treated rats. This is not found to be the case; the injected animals in a state of healing accumulate nearly twice as much Ca* or Sr* per gm. of dry bone and about 1.5 times as much per gm. of bone ash (Table II). In the vitamin D-deficient animals more of the injected Ca* and Sr* is run off into the urine (Table I). This finding is evidence that

TABLE I

Partition of Labeled Calcium and Strontium in Body and Excreta of Rachitic and Vitamin D-Treated Rats

The values are given in per cent of the total dose. They are the means ± the mean deviations of four animals in each group sacrificed 3 days after Sr* administration, and the mean values of two animals given Ca*.

	Mode of administration	Urine	Feces	Skeleton	Teeth	Residual carcass
Strontium*	Oral, no vitamin D	21.3 ± 2.1	67.0 ± 1.9	9.5 ± 2.1	1.3 ± 0.5	1.2 ± 0.45
	Oral, with vitamin D	32.6 ± 3.3	48.8 ± 1.8	15.0 ± 3.7	1.35 ± 0.1	2.1 ± 0.7
	Injected, no vitamin D	61.8 ± 2.6	21.0 ± 1.9	11.1 ± 0.4	2.6 ± 0.8	2.3 ± 0.4
	Injected, with vitamin D	48.8 ± 2.1	17.3 ± 4.4	26.0 ± 6.8	2.5 ± 0.25	4.0 ± 1.4
Calcium*	Oral, no vitamin D	19.5	60 0	15.0	2.1	3.4
	Oral, with vitamin D	29.4	32.5	31.0	3.5	3.6
	Injected, no vitamin D	44.0	18.5	28.0	6.5	3.0
	Injected, with vitamin D	25.0	18.0	45.0	6.0	6.0

vitamin D has a direct action on the mineralization of bone in rachitic animals, besides promoting healing indirectly by increasing the absorption of calcium from the digestive tract.

TABLE II

Accumulation of Radioactive Strontium and Calcium in Bone and Teeth of Rachitic and Vitamin D-Treated Animals

Values for Ca* and Sr* in bone and teeth are in per cent of the administered dose. Analysis of the bone was made on the femurs.

Bone

Oral, no vitamin D			Oral, with vitamin D			Injected, no vitamin D			Injected, with vitamin D		
Ash	Radio element		Ash	Radio element		Ash	Radio element		Ash	Radio element	
	Per gm dry bone	Per gm bone ash		Per gm dry bone	Per gm bone ash		Per gm. dry bone	Per gm. bone ash		Per gm. dry bone	Per gm. bone ash
Strontium* (6 rats)											
per cent			*per cent*			*per cent*			*per cent*		
31.4 ±5.2	3.9 ±1.0	12.5 ±4.0	34.1 ±8.0	5.6 ±1.5	16.5 ±4.3	30 ±4.7	5.4 ±1.0	18 ±2.6	37 ±8.0	9.8 ±1.7	26.5 ±6.8
Calcium* (2 rats)											
30.3	6.6	22	32.4	13.6	42	30.1	12.6	4.2	35.8	25.1	71

Teeth

	Oral, no vitamin D			Oral, with vitamin D			Injected, no vitamin D			Injected, with vitamin D		
	Dry weight	Radio element		Dry weight	Radio element		Dry weight	Radio element		Dry weight	Radio element	
		Total uptake	Uptake per gm dry weight		Total uptake	Uptake per gm dry weight		Total uptake	Uptake per gm dry weight		Total uptake	Uptake per gm. dry weight
Strontium* (3 rats)												
	gm.			*gm.*			*gm.*			*gm.*		
Incisors	0.15	0.92	6.2	0.15	0.98	6.6	0.17	1.34	8.0	0.19	1.60	8.6
Molars	0.13	0.20	1.6	0.12	0.26	2.2	0.14	0.30	2.2	0.11	0.34	3.3
Calcium* (2 rats)												
Incisors	0.14	2.1	15.1	0.17	3.5	20.6	0.17	5.5	32.3	0.15	4.2	28
Molars	0.12	0.38	3.2	0.13	0.75	6.0	0.13	1.1	8.5	0.12	1.0	8.4

The influence of vitamin D on the retention of calcium by the teeth is small in comparison with its influence on bone. This is illustrated by the data for the Ca* and Sr* uptake of the rat incisors and molars reported in Table II.

I am greatly indebted to Professor E. O. Lawrence and the staff of the Radiation Laboratory of the University of California for the supply of radioactive calcium and strontium. I am indebted to Elizabeth M. Cuthbertson and to Frances M. Troescher for assistance with the experimental work.

SUMMARY

Tracer experiments with radioactive calcium and strontium demonstrate that vitamin D promotes the absorption of calcium from the digestive tract. The data indicate that vitamin D also exerts a direct effect on the mineralization of bone in rachitic animals.

BIBLIOGRAPHY

1. Greenberg, D. M , in Annual review of biochemistry, Stanford University, **8**, 269 (1939).
2. Cohn, W. E., and Greenberg, D M , *J. Biol. Chem.*, **130**, 625 (1939).
3. Seaborg, G. T., *Chem. Rev.*, **27**, 199 (1940)
4. Copp, D. H., and Greenberg, D M , *Rev Scient. Instruments*, **14**, 205 (1943).
5. Pecher, C., *Univ. California Pub. Pharmacol.*, **2**, 117 (1942).
6. Greenberg, D. M., and Troescher, F. M., *Proc. Soc Exp. Biol. and Med.*, **49**, 488 (1942).
7. Nicolaysen, R., *Biochem J.*, **31**, 107, 122, 1086 (1937).

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THE ORIGIN OF THE REACTION OF HARDEN AND YOUNG IN CELL-FREE ALCOHOLIC FERMENTATION*

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Equation (1) of Harden and Young (1) states that 1 sugar molecule is fermented to CO_2 and alcohol, while a 2nd one is esterified to hexose diphosphate (HDP).

$$(1)\quad 2C_6H_{12}O_6 + 2R_2HPO_4 = 2CO_2 + 2C_2H_5OH + C_6H_{10}O_4(R_2PO_4)_2 + 2H_2O$$

The reason for this chemical balance was found in the "coupling reaction" of fermentation (Meyerhof, Ohlmeyer, and Möhle (2)). The coupling itself is caused by the intermediary formation of a 1,3-diphosphoglyceric acid and the following transphosphorylation with the adenylic system (Warburg and Christian (3), Negelein and Bromel (4)).

$$(2)\quad \text{3-Glyceraldehyde phosphate} + \text{phosphate} + \text{cozymase} \rightleftarrows \text{1,3-diphosphoglyceric acid} + \text{dihydrocozymase}$$

$$(3)\quad \text{1,3-Diphosphoglyceric acid} + \text{adenosine diphosphate} \rightleftarrows \text{3-phosphoglyceric acid} + \text{adenosine triphosphate}$$

Since adenosine triphosphate (ATP)[1] transfers its labile phosphate to glucose, for every mole of triose phosphate oxidized 1 mole of inorganic phosphate is taken up by the fermenting system. Besides this uptake, an internal cycle exists for the second phosphate group, which is already present in the triose phosphate and after oxidation appears finally in the phosphopyruvic acid. This phosphate is also transferred to glucose or hexose monophosphate and reappears therefore in a new molecule of triose phosphate. This internal cycle does not add new phosphate to the system in the stationary state and can therefore be omitted from the discussion.

However, another aspect of the reaction of Harden and Young is less obvious. In cell-free fermentation two phases follow each other. First there is a rapid phase, "phosphate period," for which equation (1) applies;

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[1] Throughout this paper the following abbreviations are used: HDP for hexose diphosphate, ATP for adenosine triphosphate, ADP for adenosine diphosphate, AA for adenylic acid, and apyrase for adenylpyrophosphatase. In conformity with Kalckar (5), I use the name adenylpyrophosphate for ATP and ADP, and apyrase for those dephosphorylating enzymes, which do not distinguish between both, while ATP-ase and ADP-ase may be used for the one-step enzymes.

then a second slower phase, when either the inorganic phosphate or the free sugar is exhausted, dependent on which compound was added in excess at the start. During this period the fermentation rate is controlled by the fermentation of HDP (6). In the absence of a phosphate acceptor ATP, resulting from reaction (3), can be split only by means of the enzyme adenylpyrophosphatase (apyrase).

(4) ATP = ADP + phosphate = AA + 2 phosphate

Since the highest rate obtained in cell-free sugar fermentation may approach the fermentation rate obtained with an equivalent amount of living yeast (7), and since the rate of fermentation of HDP is generally only a tenth of this or less, one may attribute the origin of the two phases to a destruction of the greater part of the apyrase of living yeast during the procedures of drying, macerating, and extracting. The apyrase would be the most sensitive and least extractable of all enzymes of fermentation.

This hypothesis, made many years ago by the author (8), was never strictly proved and while it has met with some approval (Harden (9), Lynen (10)), others have disagreed. I will not deal here with proponents of different pathways of sugar breakdown in living and non-living yeast, either non-phosphorylating fermentation in the former (Nord (11)) or a partial phosphorylation (Nilsson (12)). More serious objections could be raised against the theory of "apyrase destruction" from other observations. The complicated way in which HDP in the presence of arsenate loses its phosphate groups in the absence of apyrase (Meyerhof and Junowicz-Kocholaty (13)), simultaneously maintaining the high rate of sugar fermentation, could favor the idea that an analogous way may exist in the living cell, and that it could be destroyed by disorganization or by the various procedures for obtaining the yeast preparations. Or, one may assume the presence of a special labile enzyme in the living yeast which splits off the carboxyl phosphate without any interference of phosphate transfer (*cf.* (14) for splitting off acetyl and succinyl phosphate). Finally in the living cell transphosphorylation may be connected with the formation of higher carbohydrates and the phosphate may be split off by a reaction similar to the Cori reaction (15).

(5) Glucose-1-phosphate + glucosidic group $\rightleftharpoons$ maltosidic group + phosphate

It appeared worth while to settle this matter experimentally.

The investigation was extended in two directions: (1) a study of the behavior of apyrase of yeast and its distribution between the liquid and solid components of the cell in connection with the fermentation rate of HDP, as well as the means for preserving and separating this enzyme; (2) addition of apyrase of foreign origin to fermenting yeast preparations. Such an addition, if made in sufficient amount, should raise the rate of fermentation of HDP to the level of fermentation of free sugar, or to the "arsenate level."

Both series have led to complete verification of the hypothesis that destruction of apyrase is responsible for the two phases of cell-free fermentation.

Methods

Yeast preparations were made from Schmidt's brewers' yeast.[2]

For destruction of the yeast a modified Peirce magnetostriction oscillator with a frequency of about 8900 cycles per second, constructed and described by Chambers and Flosdorf (16), was used.[3] The apparatus has a cooling system of running water. 6 gm. of well washed, fresh yeast in 16 cc. of distilled water (or 13 cc. of distilled water + 3 cc. of 0.1 N glutathione) were exposed to sonic vibrations for 90 to 100 minutes. About three-fourths of the yeast cells were destroyed in this period of time. Cell débris was removed by centrifugation in an angle centrifuge in the cool room and the cloudy supernatant solution used.

Frozen yeast was prepared by keeping lumps of yeast in liquid air for a period of 12 to 15 hours. After melting, the mass was either diluted with water and used as such or it was centrifuged, the supernatant liquid separated, and the cell fragments washed several times with ice water. Juice and fragments served for different experiments.

ATP was prepared from rabbit muscles by B. D. Polis and myself,[4] according to Kerr's procedure (17), which gives somewhat higher yields than that of Lohmann (18). We contented ourselves with one reprecipitation of the barium salt in weakly acid solution. The air-dried salt had a total P content of 10 to 11 per cent, 63 to 67 per cent of which was 7 minute P.

Fermentation was measured with Warburg's manometric technique. Flasks of 35 cc. capacity were used with two side bulbs. Sugar or HDP was introduced in one bulb and special reagents, such as potato apyrase, in the other. The gas space was usually filled with air. If sugar is fermented at pH 6, there is practically no retention of CO_2. But this is different with HDP, in which, with the splitting off of phosphate, basic equivalents are developed (Meyerhof and Suranyi (19)). The retention of CO_2 amounts to 1.24 and all measured values of CO_2 formation must be multiplied by this factor.

Apyrase from potatoes was recently described by Kalckar (5). He purified the enzyme starting with a potato extract, which was cleared of most of the proteins by 0.6 saturation with ammonium sulfate. Probably owing to differences in the extracting procedure this method failed in my hands. I precipitated a large part of the inert protein by 0.4 saturation (30 gm. of solid salt to 100 cc. of solution). The solution was concentrated after addition of a further amount of solid salt (20 gm. per 100 cc.), until it reached full saturation. The precipitate was dissolved in water, dialyzed for 2 hours, and centrifuged. The solutions used for the manometric experiments contained 5 to 12 mg. of protein per cc., 0.1 cc. of which splits 200 to 300 γ of 7 minute P from ATP in 5 minutes at 30° at pH 6.

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[2] I thank C. Schmidt and Sons, Inc., Philadelphia, for an ample supply of fresh brewers' yeast.

[3] I thank Dr. Leslie A. Chambers of the Johnson Foundation for Medical Physics for permission to use the oscillator, and Dr. J. Conway and Miss J. Clawson for their help.

[4] Further studies on purification of apyrase will be published by us.

Protein content was determined by the biuret method (Robinson and Hogden (20)). For measuring the apyrase activity of the different preparations, the properly diluted enzyme was incubated with ATP, containing 350 to 450 γ of 7 minute P, in 1.4 cc. total volume, and the turnover measured at 30° after 5, 10, and 15 minutes. 0.1 to 0.2 cc. of 0.1 M glutathione was always added, because of its beneficial effect on all kinds of apyrase. For the yeast enzyme, veronal buffer in the optimal range of pH 9 was used; for potato apyrase, succinate-borax at pH 6.

Results

Apyrase from Yeast. Distribution in Connection with Fermentation Rate—Generally the separation of the two phases of sugar fermentation is more distinct with yeast extracts (press juice, maceration juice) than with solid preparations (dried yeast, acetone yeast, frozen yeast, etc.). According to the proposed hypothesis this should result from the unequal distribution of apyrase between the solid elements and the juice of the cell. The structural elements, containing more of the enzyme, would ferment HDP quicker, which would render the transition from the first to the second phase of sugar fermentation less marked.

This is actually the case. A good material for demonstration is yeast frozen in liquid air. When the thawed yeast is centrifuged and the solid elements removed, the juice ferments glucose easily but reacts on HDP extremely slowly. On the other hand, the solid elements washed several times with ice water, unable to ferment alone because of lack of coenzymes, show a high rate of fermentation of HDP after addition of the pure coenzymes or of boiled yeast juice.

The same volume of washed fragments splits ATP 6 times as fast as the juice, as *e.g.* in Table I, Protocol M177, where the HDP fermentation rate of the juice is 19.0 per cent that of the fragments (with boiled juice), the apyrase activity 15.5 per cent (without boiled juice). In Table I a number of experiments are reproduced with different yeast preparations. The mole turnover of HDP, measured by CO_2, and of ATP measured by 7 minute P split off in a parallel sample under similar conditions, is very nearly the same in all cases.

It follows from the above experiments that apyrase of yeast is an enzyme bound to the cell structure. Supersonic vibration often brings such enzymes into solution; the same is true for apyrase. After 90 to 100 minutes oscillation of yeast, two layers are obtained in an angle centrifuge, a supernatant cloudy solution and a precipitate consisting of cells and débris. This precipitated fraction is rather inactive, nearly all activity being found in the cloudy suspension. But here again it is not the suspended particles, but the solvent which contains the enzymatic activities of fermentation. The particles can be removed by centrifuging at 10,000 R.P.M., with little change in fermenting power. However, since high speed centrifuging was generally not available, the cloudy supernatant was used directly in most of the experiments.

TABLE I

Fermentation Rate of HDP and Dephosphorylation of ATP by Same Yeast Preparations

Protocol No.	Kind of preparation	Material used	Temperature	Time	HDP fermentation		7 minute P from ATP	Micromole turnover		
								HDP		ATP
								CO_2	P	P
			°C	min	c mm CO_2	split mg P	mg P			
M165	Dry yeast	1 cc. total	25	60	155	0 20	0.23	6.9	6.5	7.4
				190	481	0.675	0.60	21.5	21.8	19.5
M177	Frozen in liquid air	0.4 cc. washed fragments	22	15			0.44			
				30	460		0.76	20.5		24.5
		0.4 cc. juice	22	30	88		0.118	3.9		3.8
M178	" "	0 4 cc. total solution	22	60	532		0.556	23.8		18.0
		0.6 cc. washed fragments	22	60	393		0.444	16.8		14.2
M182	Supersonic	0.7 cc. total	22	60	372		0.52	16.6		16.8
		0 7 cc. supernatant	22	60	361		0.51	16.1		16.5
M189	" "	0.6 cc. total	22	15	200		0.258	8.9		8.3
		0.6 cc supernatant	22	15	160		0.251	7.2		8.1

The rate of fermentation of HDP is about 20 per cent of the maximal rate of glucose. The rate of this fermentation agrees with the speed of dephosphorylation of ATP. Often a complete quantitative agreement is obtained (Table I, Protocols M182 and M189).

Some Purification Steps of Dissolved Apyrase from Yeast—The enzyme dissolved by supersonic treatment is highly unstable. Even if kept in the ice box, the apyrase activity diminishes in some hours to a fraction of the initial activity. Various means were found to stabilize the enzyme in connection with some purification. The beneficial influence of glutathione has already been mentioned. Glutathione was added to the yeast before vibration and again to the test solution, so that the final concentration was about 2×10^{-2} M. The optimum pH is 9, outside the range of most other fermentation enzymes of yeast. By making use of this fact, a clear solution of relatively high activity could be obtained by mixing the supersonic solution with the same amount of 10 per cent bicarbonate, and centrifuging. But the stability of this cleared solution was not very much improved. A better procedure is the addition of ethyl alcohol to a concentration of 10 to 15 per cent. This forms a precipitate of the suspended particles and part of the dissolved protein and leaves the enzyme in solution. With 15 per cent alcohol nearly 50 per cent of the total protein is removed and only 10 per cent of the total activity lost. Adding to the solution a tenth of its volume of a concentrated suspension of $Al(OH)_3$ and centrifuging remove a further 30 to 40 per cent of the remaining protein.[5] This solution is now sufficiently stable for use during 24 hours with a small loss of activity. Even if kept for a week in the ice box, only a third of the activity is lost. Such a solution has an activity of $Q_P = 250$ at 30°,[6] compared with 50 to 100 for the supersonic solution under optimal conditions, or with 20 to 30 in the pH range of fermentation.

Some Properties of Dissolved Apyrase from Yeast—Apyrase from yeast exhibits the same pH optimum as apyrase from muscle and also an activation by some bivalent cations. But Ca, which strongly activates ATPase of myosin (21), has here a depressing effect. Mn, however, activates apyrase of yeast, while Mg seems ineffective. Since the solution already contains the metal components of the yeast, an exact picture of the activating ions cannot be obtained in this way. An especially interesting feature is the high sensitivity to sodium azide, while KCN is ineffective. This inhibition is much stronger at pH 9 than at pH 6, at which the inhibition of hemin catalysis by azide occurs. 2×10^{-4} N azide inhibits about 60 per cent, 1×10^{-4} 40 per cent. The inhibition is not quite the same with different preparations, but is of the same order of magnitude. On the other hand, the inhibition is not complete even with much higher concentrations. Absence of glutathione does not change the inhibition by azide, and changing the concentration of Mn also has no appreciable effect. Since KCN is ineffective, it is improbable that iron is the responsible metal for the azide inhibition. Neither myosin nor potato apyrase is affected by azide (Table II).

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[5] Similar steps were used by Kalckar at a higher level of purity for potato apyrase.

[6] Q_P calculated like Q_{CO_2} (31 γ of P = 22.4 c.mm.), Q = c.mm. turnover per 1 mg. of dry weight in 1 hour.

Increase of Fermentation Rate of Hexose Diphosphate to "Arsenate Level" by Addition of Apyrase from Potatoes—If the slow rate of fermentation of HDP compared with sugar is due to a preferential destruction or removal of apyrase in the yeast preparations, then addition of enough apyrase from other sources must raise the fermentation rate to that of sugar or to the arsenate level. In 1934 it was shown (6) that under optimal conditions the arsenate rate (with 2 × 10^{-3} M arsenate) equals or surpasses the maximum rate of sugar fermentation in the phosphate period. Some time later it was found (22) that addition of creatine together with the enzyme fraction of muscle, containing the transphosphorylase for creatine, raises the speed of fermentation of HDP in maceration juice to the arsenate level. This experiment proves that the turnover of HDP in the presence of a phosphate acceptor conforms to that necessary for a true intermediary of sugar breakdown. Now it will be shown that the same happens in the absence of a stoichiometric amount of phosphate acceptor by a sufficiently high concentration of apyrase. This result was achieved with the help of purified apyrase from potatoes. The advantage of this enzyme compared with others, especially with myosin, is its high stability and concentration, its favorable optimal pH range at 6 (close to the optimum of fermentation), and its easy solubility in water. Kalckar has obtained purified preparations, which split 5 to 7 γ of P in 5 minutes at 30° per microgram of protein. My own less purified preparations with an activity of only 10 per cent of this, are nevertheless, per cc., at least 10 times more active than the most

TABLE II

Inhibition of Adenylpyrophosphatase from Yeast by Azide

Protocol No	Purification step	Glutathione added	Mn added	Time	Azide	P split	Per cent inhibition
				min	M	γ	
P449	15% alcohol	+	+	10		148	
		−	+	10		140	
		−	+	10	5.5 × 10^{-4}	57	59
		−	+	10	5.5 × 10^{-3}	20	86
P455	$Al(OH)_3$	+	+	5		123	
		−	+	5	1.0 × 10^{-4}	77	38
		−	+	5	1.8 × 10^{-4}	35	71
		+	+	5	1.8 × 10^{-4}	40	67
P467	"	+	+	5		127	
		+	−	5		109	
		+	−	5	1.8 × 10^{-4}	30	72

concentrated solutions of myosin apyrase described in the literature or obtained in this laboratory by Dr. Polis.

When added directly to HDP, this fraction brings about a very slow dephosphorylation, because of contamination with an ordinary phosphatase, but if added to a suitable yeast preparation, it increases enormously the rate of fermentation of HDP, and if added in sufficient amount raises it to the arsenate level or somewhat above. If added together with arsenate, it has no effect (Table III, Protocol M285). This result is more regularly obtained if some ATP or AA is added at the same time. Addition of ATP alone has not the slightest effect, but in the presence of a large amount of apyrase it gives a marked increase in speed (Fig. 1, Curves II and III). These experiments prove our hypothesis. The results must be expected if the factor which is lacking in the system is apyrase and nothing else.

TABLE III

Maximal Rates of Fermentation of Glucose (G.) and HDP (H.)

Protocol No	Preparation	Temperature	Sugar	Addition	Potato apyrase 10^{-3} units	CO_2 in 3 min	Multiple of direct fermentation	Q_{CO_2}
		°C.				c.mm.		
M284	Supersonic, 22 mg. dry weight	25	H.			27		
			"	ATP		27		
			"	AA		56		
			"	ATP	160	96	3.5	
			"	AA	160	84		
			"	Arsenate		120	4.5	
M285	" "	27	G.			54		
			"	Arsenate		96		
			H.	ATP		31		
			"	"	130	134	4.3	130
			"	Arsenate		143		
			"	"	130	150	4.9	
M287	" "	27	"			22		
			"	ATP	310	175	8	158
			"	"	130	125		
			"	Arsenate		172	8	
M290a	Frozen yeast, total 41 mg dry weight	29	"			81		
			"	ATP	240	225	2 8	110
			"	Arsenate		194	2 4	
M290b	Frozen yeast juice, 39 mg. dry weight	29	G.			114		
			"	Arsenate		112		
			H			11		
			"	ATP	240	160	15	82
			"	"	120	112		
			"	Arsenate		133	12	

In this case, addition of ATP alone cannot have an effect because the enzyme is saturated with it. But if a great excess of enzyme is added, the situation is different. Now the enzyme is no longer necessarily saturated by the preexisting amount of adenine nucleotide. It is moreover interesting to note that the highest possible rate obtained with an excess of enzyme is a little more than the arsenate level (Figs. 2 and 3, and Table III). Now another enzymatic system, and not the apyrase, controls the speed.

In order to obtain this result, the added amount of apyrase, measured in terms of 7 minute P split at 30° under optimal conditions, must be about 3 times as high as the amount of HDP fermented at the arsenate level.

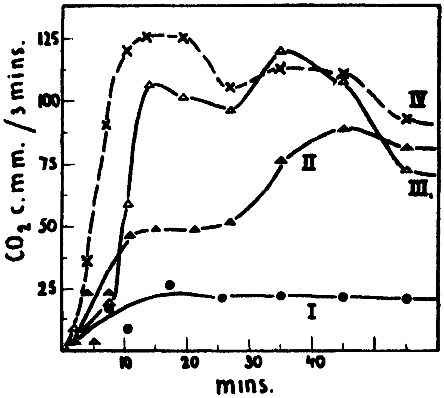

FIG 1 Rate of fermentation of HDP in supersonic solution (Protocol M285). Curve I, without additions; Curve II, + 0.7 cc of potato apyrase; Curve III, + 0 7 cc of potato apyrase + 0 2 cc of ATP (280 γ of 7 minute P); Curve IV, + arsenate (2 5 × 10^{-3} M) All samples contain 0 5 cc of supersonic yeast solution, 0.4 cc of boiled juice, 0 1 cc of 0 1 N glutathione, 1 × 10^{-3} M $CaCl_2$, 5 × 10^{-4} M $MnSO_4$, 0 7 cc of HDP with 3 0 mg of P, introduced from one side bulb in the beginning; the above additions made 6 minutes later from the other side bulb Volume of fluid 2.6 cc , 27°.

Besides the somewhat lower temperature of the fermentation experiments (28° and 25°), this apparently is due to a lowered activity of the apyrase in the fermenting mixture. Especially the Ca ion concentration is here suboptimal for potato apyrase, since otherwise it would inhibit the fermentation.

Example—In Protocol M287 (Fig. 2) the maximal speed is 172 c.mm. of CO_2 per 3 minutes or 4.9 mg. of P per hour. The arsenate level is reached by 0.7 cc. of potato apyrase, which splits under optimal conditions 19 mg. of 7 minute P per hour. But with 0.3 cc. of an apyrase solution of similar strength, which splits 8 mg. of 7 minute P per hour, the curve does not reach the arsenate level, but remains at 125 c.mm. of CO_2 per minute. The

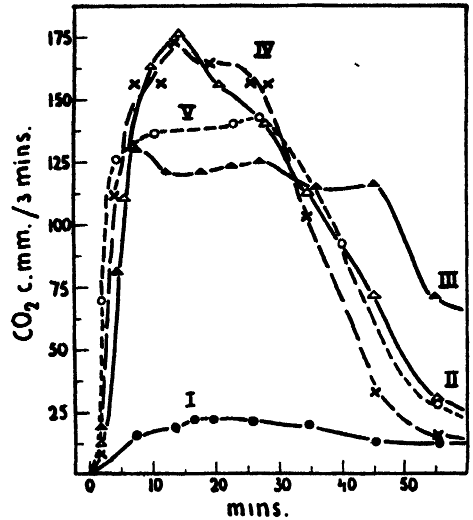

FIG. 2. Rate of fermentation of HDP and glucose in supersonic solution (Protocol M287). Curve I, HDP without additions; Curve II, HDP + 0.7 cc. of potato apyrase + ATP; Curve III, HDP + 0.3 cc. of potato apyrase + ATP; Curve IV, HDP + arsenate (2.5×10^{-3} M); Curve V, glucose (12 mg.) + arsenate. Other components are like those in Fig. 1.

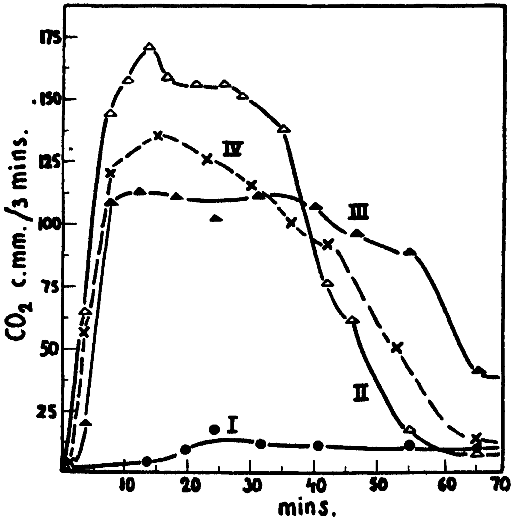

FIG. 3. Rate of fermentation of HDP in juice of frozen yeast (Protocol M290b). Temperature 29°. Curve I, HDP, without additions; Curve II, + 0.6 cc. of potato apyrase + ATP; Curve III, + 0.3 cc. of potato apyrase + ATP; Curve IV, + arsenate. All samples contain 0.6 cc. of yeast juice and the other components as in Fig. 1.

same is true in the other experiments. In Protocol M290 (Fig. 3) the arsenate level corresponds to 136 c.mm. of CO_2 per 3 minutes or 3.76 mg. of P per hour. With potato apyrase splitting 14.2 mg. of 7 minute P per hour, 170 c.mm. of CO_2 per 3 minutes are obtained, a figure appreciably above the arsenate value. However, with half the amount of apyrase, only 116 c.mm. of CO_2 per 3 minutes are obtained, which is below the arsenate level. For comparison the content of apyrase in the solution is expressed in units, 1 unit being the amount of enzyme which splits 1 mg. of P per minute at 30° under optimal conditions (Table III).

While added ATP alone is without effect on the rate of fermentation of HDP, AA, as shown by Ohlmeyer (23), increases the rate because it serves as P acceptor. Its effect corresponds in amount and duration to the phosphorylation of AA to ATP. Simultaneously some of the HDP is split to hexose monophosphate, in the reaction

$$\text{AA} + 2\text{HDP} = \text{ATP} + 2 \text{ hexose monophosphate} \tag{6}$$

This reaction, in going to the right, requires energy and is coupled with the oxidation-reduction step of fermentation. But the hexose monophosphate generated in this way has apparently no great influence on the fermentation speed. If potato extract is added together with AA, the system, after complete phosphorylation of the AA, is identical with that formed by addition of ATP and potato extract and behaves similarly (Table III, Protocol M284).

Very suitable preparations to demonstrate these effects are the yeast suspensions after supersonic vibration, cleared from cells and débris by centrifuging, or yeast frozen in liquid air and centrifuged after thawing to obtain the clear juice. With supersonic solutions the arsenate level is about 5 to 8 times the rate of HDP fermentation in the absence of arsenate, and the increase with excess of potato apyrase corresponds to this relation. With frozen yeast, in which most of the yeast apyrase remains bound to the solid particles, the arsenate level of the full suspension (particles + juice) is only 3 times the ordinary rate. But after the solid cell material is removed by centrifuging, the rate of HDP fermentation is very low, and now a spectacular increase of speed of about 15 times occurs with the potato enzyme as well as with arsenate (Fig. 3).

These ratios must not be taken as rigidly fixed. They depend somewhat on the dilution of the extract and on the amount of coenzymes added for "fortifying" the enzymatic system. In the supersonic solutions the yeast was diluted during the vibration and again afterwards, so that the concentration of the soluble substances became one-twentieth of that in the living yeast. Most often some boiled juice was added in the fermentation experiments, but even with this addition the dilution was still about one-twelfth that of living yeast. Addition of cozymase had little effect and was generally omitted. The absolute speed would be considerably increased if the dilution were less, but it was not advisable to have such high speeds for exact measurements; nor was it feasible to have higher concentrations for effective destruction of the yeast by vibration. Even under these relatively unfavorable conditions the maximal fermentation rate of HDP in the presence of potato apyrase amounts to 40 to 50 per cent of that of free sugar for the same amount of living yeast (Q_{CO_2} about 150 at 28°, instead of 300 to 350 for living yeast). For cell-free juice of frozen yeast the figure is about $Q_{CO_2} = 80$ and for total frozen yeast $Q_{CO_2} = 100$. In all samples some acetaldehyde (about 1 mg.) was introduced together with the HDP to shorten the induction period. For the same reason the glucose solution added contained acetaldehyde as well as a trace of HDP (24).

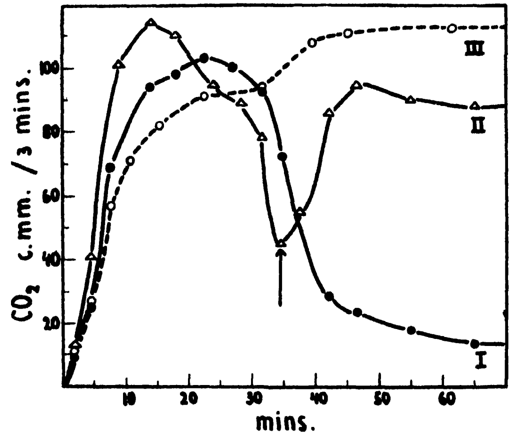

Fig. 4. Rate of fermentation of glucose in juice of frozen yeast (Protocol M290b). Temperature 29°. Curve I, without additions; Curve II, the same, but at arrow (36 minutes) 0.5 cc. of potato apyrase added; Curve III, arsenate (2.5×10^{-3} M). All samples contain 12 mg. of glucose, with HDP (0.2 mg. of P) and 0.5 mg. of acetaldehyde. Other components are like the experiments in Fig. 1 The peak of Curve II comes earlier than that of Curve I and is higher, because the enzyme mixture of Curve II is somewhat more concentrated before the addition of 0.5 cc. of potato extract. Afterwards the concentration of both samples is the same.

For ascertaining that transphosphorylation plays no part in the high rate of HDP fermentation, changes of inorganic phosphate were determined simultaneously with CO_2 in several experiments. The ratio, mole of H_3PO_4 split to mole of CO_2, was always close to 1, with the high turnover in the presence of potato apyrase. A great deviation from this ratio occurs only at a very slow fermentation because of higher carbohydrates (preexisting in yeast juice), which can be phosphorylated by means of the

Cori reaction, thus diminishing the amount of free phosphate. For example, in Protocol M287 (Table III) the microequivalents of phosphate split in 1 hour were 91, 93, and 3, for HDP + potato apyrase, HDP + arsenate, and HDP without addition, while the respective CO_2 microequivalents were 89, 86, and 15.

The high rate of fermentation is maintained until the HDP is nearly exhausted. The amount added was about 90 microequivalents (2.8 mg. of P).

In general it is difficult to carry out the same experiments with glucose instead of HDP. Here, too, it could be presumed that when potato enzyme would be added from the start of the fermentation the system would behave as in the presence of arsenate; so that the maximum rate attained during the phosphate period would be upheld until all glucose is fermented. But apparently on account of the impurities which accompany the potato apyrase this does not happen. The extract interferes considerably during the phosphate period with the fermentation of glucose. Theoretically only half of the amount of apyrase should be sufficient for the maximal fermentation rate of glucose as compared with that of HDP, since for 2 sugar molecules only 2 phosphates have to be removed by apyrase. This expectation cannot be tested for the reasons given above. But if one waits until the peak of the fermentation rate of glucose is passed before introducing the potato extract, then the rate of fermentation rises again to the arsenate level, because in this second period the splitting of HDP controls the rate (Fig. 4).

All these facts show conclusively that the two phases have originated from a relative deficit of apyrase in the dead yeast preparations.

DISCUSSION

The glucose molecule passes anaerobically through twelve stable intermediary stages before becoming alcohol and CO_2; at least three dissociable organic coenzymes, twenty or more enzyme proteins, and some bivalent metals (Mn, Mg) are necessary for the breakdown. Since every single reaction concerned may be varied in speed according to the dilution of the coenzymes and other reactants and to the activity of the enzyme proteins, it is small wonder that many variations occur in yeast preparations in regard to the speed with which glucose and HDP ferment, or in regard to the amount in which the mono- or diesters of sugar accumulate. But in spite of this, the separation of the cell-free fermentation of sugar into two more or less distinct phases of very different speed, the phosphate period and the HDP period are a customary feature.

The most extensive work devoted to this problem is that of Nilsson (12), who, between 1936 and 1942, investigated yeast preparations which either showed the usual break when half of the sugar was fermented or did not show it (*intakte Trockenhefe*[7]).

In the latter case addition of cytolytic agents, dyestuffs, etc., induced the appearance of this break. Summarizing his numerous papers in a lecture (25) given in Heidelberg at the same place where the modern scheme of sugar fermentation was developed, Nilsson rejected this scheme because of these findings. The break according to this author proves the decomposition of the hexose into one phosphorylated and one unphosphorylated half which ferment in the living yeast with equal speed. After an organizing principle in the living yeast is destroyed, only the unphosphorylated half ferments in yeast preparations, while the other half "abnormally" condenses to HDP.[8]

In the present paper it is shown that this accumulation of HDP is due to a relative deficit of apyrase, which is the most sensitive enzyme of fermentation in yeast and therefore more damaged than the others. All observations of Nilsson are easily explained by the greater or smaller destruction of the yeast apyrase in his different preparations. Of course fermentation of glucose, and HDP in the absence of glucose, cannot be compared quite strictly. Very small differences of the concentrations of coenzymes, of the pH, and of the total salt present can have a great influence on the speed of fermentation. In glucose fermentation the phosphate diminishes, in fermentation of HDP it rises, and the pH changes in both cases differently. Nevertheless, in all systems investigated here, the highest rate obtained with HDP and excess of potato apyrase was at least the same, but mostly higher, than the highest rate obtained with glucose. A better comparison is given by the fermentation of HDP in presence of arsenate, because this fermentation is independent of apyrase. With addition of enough apyrase this arsenate level is always reached or exceeded.

SUMMARY

The two phases of cell-free alcoholic fermentation of yeast, the fast "phosphate period" and the slow "hexose diphosphate period," result from a destruction of the sensitive and structurally bound adenylpyrophosphatase of the living yeast by the drying and extracting procedure.

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[7] A sharp break just after fermentation of half of the sugar is to be expected if, at the time when all sugar has disappeared by fermentation and esterification, inorganic phosphate is still available. If, on the contrary, all of the inorganic phosphate is esterified before the free sugar is exhausted, two breaks occur, one at the time when the phosphate disappears and a second one when the free sugar disappears. Then for two phosphate groups liberated from the ester only one hexose ferments, instead of two. Actually for many reasons, including a partial formation of hexose monophosphate besides diphosphate, the breaks are often rather indistinct; so that from the mere aspect of the curves, which is the sole criterion used by Nilsson, it cannot always be decided what caused the break.

[8] For further discussion, refer to the author's review (26).

Two series of experiments prove this thesis. (1) In different preparations of yeast the adenylpyrophosphatase remains mainly with the solid elements. Fermentation of hexose diphosphate runs parallel to this distribution and the rate of fermentation agrees closely with the activity of the adenylpyrophosphatase of the same preparation. (2) By addition of concentrated adenylpyrophosphatase of foreign origin (potatoes) to fermenting yeast juice, the fermentation rate of hexose diphosphate is increased to the maximal rate of sugar fermentation, or to or above the "arsenate level."

Some properties of adenylpyrophosphatase of yeast are described. Supersonic vibration brings a large part of the enzyme into solution. The dissolved enzyme can be partially purified, and is stabilized by purification. It is highly sensitive to sodium azide, 2×10^{-4} N inhibits over 50 per cent but it is insensitive to KCN.

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BIBLIOGRAPHY

1. Harden, A., and Young, W. J., *Proc. Roy. Soc. London, Series B*, **80**, 299 (1908).
2. Meyerhof, O., Ohlmeyer, P., and Mohle, W., *Biochem. Z*, **297**, 90, 113 (1938).
3. Warburg, O., and Christian, W., *Biochem. Z.*, **303**, 40 (1939).
4. Negelein, E., and Brömel, H., *Biochem. Z.*, **303**, 132 (1939).
5. Kalckar, H. M, *J. Biol. Chem.*, **153**, 355 (1944).
6. Meyerhof, O., *Biochem. Z.*, **273**, 80 (1934).
7. Harden, A., Alcoholic fermentation, Monographs on biochemistry, London, 4th edition (1932)
8. Meyerhof, O., *Ergeb Physiol*, **39**, 10 (1937).
9. Harden, A, Hyllningskrift till Bertil Almgren, Stockholm, 313 (1938).
10. Lynen, F., *Ann. Chem*, **539**, 1 (1939)
11. Nord, F., Hofstetter, H., and Dammann, E., *Biochem. Z.*, **293**, 231 (1937).
12. Nilsson, R., and Alm, F., *Biochem Z.*, **286**, 254, 373 (1936); **304**, 285 (1940); **308**, 262 (1941).
13. Meyerhof, O., and Junowicz-Kocholaty, R, *J. Biol. Chem.*, **145**, 443 (1942).
14. Lipmann, F, and Tuttle, L C., *J. Biol. Chem.*, **153**, 571 (1944).
15. Cori, C. F, Cori, G. T, and Green, A A., *J. Biol. Chem*, **151**, 39 (1943)
16. Chambers, L. A., and Flosdorf, E. W., *Proc. Soc. Exp. Biol. and Med.*, **34**, 631 (1936).
17. Kerr, S. E., *J. Biol. Chem.*, **139**, 121 (1941).
18. Lohmann, K., *Biochem. Z.*, **254**, 381 (1932).
19. Meyerhof, O., and Suranyi, J., *Biochem. Z.*, **178**, 427 (1926).
20. Robinson, H. W., and Hogden, C. G., *J. Biol. Chem.*, **135**, 707 (1940).
21. Bailey, K., *Biochem. J.*, **36**, 121 (1942).
22. Meyerhof, O, Schulz, W., and Schuster, P. H., *Biochem. Z.*, **293**, 309 (1937).
23. Ohlmeyer, P., *Biochem. Z.*, **283**, 114 (1935).
24. Meyerhof, O., *Z. physiol. Chem.*, **102**, 185 (1918).
25. Nilsson, R., *Naturwissenschaften*, **31**, 23 (1943).
26. Meyerhof, O., *Ann. New York Acad. Sc.*, **45**, 357 (1944).

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MICROBIOLOGICAL ACTIVITY OF SYNTHETIC BIOTIN, ITS OPTICAL ISOMERS, AND RELATED COMPOUNDS

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In a previous report, synthetic and natural biotin were stated to have identical growth-promoting properties for *Lactobacillus arabinosus* 17-5 (1). This paper gives a more detailed comparison of these substances in the growth of four other biotin-requiring microorganisms. The activities of synthetic *dl*- and *l*-biotin, *dl*-allobiotin, *dl*-diamino acid sulfate, desthiobiotin,[1] and the probable biological transformation of the latter to biotin are also presented.

During the writing of the present paper, data appeared in print on the conversion of desthiobiotin to biotin by yeast (3) and on the ability of the former to replace biotin in the nutrition of various microorganisms (4). Results reported here confirm and extend those already published.

The organisms used included three bacteria, *Lactobacillus casei* (5), *Lactobacillus arabinosus* 17-5 (6), and *Rhizobium trifolii* 209 (7); a yeast, *Saccharomyces cerevisiae* F6.4 (8); and a fungus, *Neurospora sitophila* 299 (9). Several other yeasts were also tested for their ability to grow with desthiobiotin. The media and procedures were, with slight modifications, those described in the literature cited.

Synthetic biotin was as potent as the natural vitamin for *Lactobacillus casei* and *Lactobacillus arabinosus* (Table I). *dl*-Biotin was only 50 per cent active, which would be expected if the *l* isomer were inert. However, *l*-biotin when used in large amounts supported full growth of both bacteria. This is probably due to incomplete separation from the active form, since its potency varied with different crystalline preparations. Contamination with traces of *dl*-biotin may also explain the small degree of activity of *dl*-allobiotin.

Semiquantitative experiments with *Rhizobium trifolii*, *Saccharomyces cerevisiae*, and *Neurospora sitophila* gave results similar to those obtained with the lactobacilli.

Synthetic biotin, like the natural product, combined readily with avidin and could be liberated from the latter by heat.

Heating biotin with alkali, under the proper conditions, splits the urea ring with loss of the CO group (10). The diaminocarboxylic acid thus formed has 10 per cent (molar basis) the activity of biotin for *Saccharomyces cerevisiae* (11). This compound,[2] obtained synthetically as *dl*-diamino acid sulfate, was 3.6 per cent and 7.2 per cent as potent as biotin for *Lactobacillus arabinosus* and *Lactobacillus casei*, respectively. These figures are based on the assumption that only the *d* form is active, since only the *d* form of the complete vitamin is active. This further emphasizes the importance of the urea ring for the full biological activity of biotin.

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[1] We are indebted to Dr. S. A. Harris for the sample of desthiobiotin and to Dr. J. C. Keresztesy for a sample of natural biotin. For the other compounds refer to Harris *et al.* (2).

TABLE I

Activity of Synthetic Biotin and Stereoisomers Compared to Natural Biotin

Compound	Per cent activity	
	Lactobacillus casei	*Lactobacillus arabinosus*
Biotin	104	101
dl-Biotin	51	52
l-Biotin	0.024	0.019, 0.075, 0.030, 0.006*
dl-Allobiotin	0.003	0.002

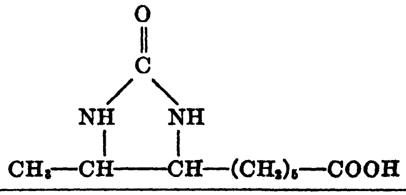
* Each value is for a different crystalline preparation.

TABLE II

Growth of Biotin-Requiring Microorganisms with Desthiobiotin

Organism	Growth
Lactobacillus casei	−
" *arabinosus* 17-5	−
Rhizobium trifolii 209	−
Neurospora sitophila 299	+
Saccharomyces cerevisiae F6.4	+
" " 139	+
" " Gebrüder Mayer	+
Schwanniomyces occidentalis 116	+

Replacement of the sulfur atom in biotin by 2 hydrogen atoms (12) also profoundly affects microbiological activity. The resulting compound, desthiobiotin, promoted growth of *Saccharomyces cerevisiae* 139 as well



$$\begin{array}{ccccc} & & \mathrm{O} & & \\ & & \Vert & & \\ & & \mathrm{C} & & \\ & \diagup & & \diagdown & \\ & \mathrm{NH} & & \mathrm{NH} & \\ & | & & | & \\ \mathrm{CH_3{-}} & \mathrm{CH} & \mathrm{{-}{-}{-}} & \mathrm{CH} & \mathrm{{-}(CH_2)_5{-}COOH} \end{array}$$

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[2] Kindly supplied by Dr. A. C. Shabica.

as biotin but was inactive for *Lactobacillus casei* (13). Desthiobiotin could be substituted for biotin in the growth of all yeasts so far tested (Table II) and also in the nutrition of the fungus, *Neurospora sitophila*. However, the bacteria were unable to utilize it.

Saccharomyces cerevisiae F6.4 developed slowly with desthiobiotin. Little or no growth was evident, usually, for 1 or 2 days, although ultimately it equaled that obtained with biotin. The lag did not occur when a desthiobiotin-grown culture was subcultured in desthiobiotin medium. This was demonstrated in the following experiment. Cultures of the yeast, previously grown with biotin and with desthiobiotin, were plated out on molasses agar medium. Portions of single colonies from plates of each type of medium were inoculated into liquid biotin and desthiobiotin media. Growth was recorded after incubation for 1 day. Colonies derived from the biotin culture grew well with biotin and poorly with desthiobiotin, whereas those from the desthiobiotin culture developed equally well with both compounds (Table III).

TABLE III

Growth of Saccharomyces cerevisiae F6.4 with Biotin and with Desthiobiotin As Influenced by Previous Contact with These Substances

Colony No.	Biotin culture		Desthiobiotin culture	
	Inoculated into media containing			
	Biotin	Desthiobiotin	Biotin	Desthiobiotin
1	66	92	82	84
2	78	95	80	81
3	69	93	79	79
4	78	94	78	81

The results are given as per cent transmissible light measured in an Evelyn photoelectric colorimeter; uninoculated medium = 100.

There are two possible explanations for the slow development of this strain of yeast when cultivated for the first time with desthiobiotin. Either the cells require time to "adapt" themselves to desthiobiotin, *i.e.* to synthesize the enzymes necessary for its utilization, or else only a small number of the cells in the inoculum are able to grow with desthiobiotin and, consequently, more time is required for these few variants to produce full growth. When a portion of a molasses agar slant culture of the yeast was plated out with synthetic agar media containing biotin and desthiobiotin, respectively, an equal number of colonies were obtained on both media and the colonies on desthiobiotin agar were much smaller than those on the biotin medium. The slow development of *Saccharomyces cerevisiae* F6.4 in desthiobiotin medium is due, therefore, to the gradual "adaptation" of the cells to desthiobiotin and not to the selective growth of variant cells. Cultures adapted to grow rapidly with desthiobiotin lost this ability when subcultured once or twice with biotin.

It was presumed that microorganisms which develop with desthiobiotin are able to resynthesize the sulfur ring to form biotin. Lack of such synthetic powers in the bacteria would explain their inability to grow with desthiobiotin. The former is highly probable since HCl extracts of *Saccharomyces cerevisiae* F6.4 grown with desthiobiotin supported full growth of biotin-requiring *Rhizobium trifolii*, *Lactobacillus casei*, and *Lactobacillus arabinosus*, none of which can utilize desthiobiotin, as previously shown. Furthermore, treatment of such extracts with Raney's nickel catalyst, which is known to convert biotin to desthiobiotin by removal of sulfur (12, 13), destroyed their activity for *L. casei and L. arabinosus* but not for *S. cerevisiae*, thus apparently completing the cycle of desthiobiotin $\xrightarrow{\text{yeast}}$ biotin $\xrightarrow{\text{Raney Ni}}$ desthiobiotin. Also, as with biotin, the activity of the yeast extract was neutralized by avidin.

TABLE IV

Effect of Desthiobiotin Concentration on Formation of Biotin by Saccharomyces cerevisiae F6 4

Desthiobiotin per 100 cc medium	Growth (turbidity)*	Biotin formed per 100 cc culture	Per cent conversion
γ		γ	
0.01	56	0.01	100
0.1	38	0.066	66
1.0	39	0.26	26
10.0	37	0.23	2.3
100.0	37	0.20	0.2

* Per cent transmissible light.

When *Saccharomyces cerevisiae* F6.4 was grown with 0.1 γ of desthiobiotin per liter of medium, 0.11 γ was found as biotin, by assay with *Lactobacillus casei*, in the HCl extract of the cells, autoclaved at 15 pounds for 2 hours. Only 30 per cent of the biotin activity was extractable with water, indicating that most of the vitamin is present in a bound form. It is noteworthy that all of the biotin was within the cells; in no instance was there more than 5 per cent in the external medium.

Maximum growth of the yeast was obtained with 0.1 γ of desthiobiotin per 100 cc. of medium, whereas maximum conversion, 4 times that with 0.1 γ, occurred with 1.0 γ of desthiobiotin (Table IV). This would seem to indicate, in contrast to the conclusions of Dittmer, Melville, and du Vigneaud for Strain 139 (3) that more biotin is formed by the cells than is necessary for growth.

By replacing all of the sulfate salts in the basal yeast medium with equivalent chlorides, it was possible to determine the influence of different sources of sulfur on the transformation of desthiobiotin. 4-Methyl-5-β-hydroxyethylthiazole, cystine, and methionine as well as sodium sulfate can supply the sulfur necessary for the conversion (Table V). Methionine supported as much yeast growth and biotin formation as sulfate, whereas little growth or conversion occurred with thiazole or cystine. Apparently, the form of the sulfur available to the yeast influences synthesis of biotin only indirectly through its quantitative effect on growth.

Conversion of desthiobiotin to biotin has been established also for the fungus *Neurospora sitophila* and probably occurs with other microorganisms which can grow with desthiobiotin.

TABLE V

Effect of Source of Sulfur on Conversion of Desthiobiotin by Saccharomyces cerevisiae F6.4*

Source of sulfur	Per 100 cc. medium	Growth†	Biotin formed per 100 cc culture	Per cent conversion
	mg.		γ	
No sulfur		89	0.0015	0.15
4-Methyl-5-β-hydroxyethylthiazole	20	77	0.01	1.0
Cystine	20	59	0.025	2.5
Methionine	20	25	0.29	29.0
Sulfanilamide	5	86	0.001	0.1
Sodium sulfate	20	23	0.28	28.0

* 1 γ of desthiobiotin per 100 cc. of medium.

† Per cent transmissible light.

SUMMARY

Comparison of the growth-promoting properties of synthetic biotin and natural biotin for five representative microorganisms indicates that the two substances are identical. The synthetic vitamin, like the natural product, forms an inactive heat-dissociable complex with avidin. *l*-Biotin and *dl*-allobiotin support microbial growth only when used in very large amounts; their slight activities are considered to be due, probably, to contamination with biotin or *dl*-biotin.

Synthetic *dl*-diamino acid sulfate is 4 to 7 per cent as active as biotin for lactobacilli.

Desthiobiotin can substitute for biotin in the nutrition of a number of yeasts and a fungus but does not support growth of the three bacteria tested. For one yeast strain, a period of adaptation to desthiobiotin is necessary before rapid growth can occur. Growth with desthiobiotin is accompanied by the formation of a substance having the properties of biotin.

BIBLIOGRAPHY

1. Harris, S. A., Wolf, D. E., Mozingo, R., and Folkers, K., *Science*, **97**, 447 (1943).
2. Harris, S. A., Wolf, D. E., Mozingo, R., Anderson, R. C., Arth, G. E., Easton, N. R., Heyl, D., Wilson, A. M., and Folkers, K., *J. Am. Chem. Soc.*, **66**, 1756 (1944). Harris, S. A., Mozingo, R., Wolf, D. E., Wilson, A. N., Arth, G. E., and Folkers, K., *J. Am. Chem. Soc.*, **66**, 1800 (1944).
3. Dittmer, K., Melville, D. B., and du Vigneaud, V., *Science*, **99**, 203 (1944).
4. Lilly, V. G., and Leonian, L. H., *Science*, **99**, 205 (1944).
5. Landy, M., and Dicken, D. M , *J. Lab. and Clin. Med.*, **27**, 1086 (1942).
6. Snell, E. E., and Wright, L. D., *J. Biol. Chem.*, **139**, 675 (1941).
7. West, F M., and Wilson, P. W., *Enzymologia*, **6**, 162 (1940).
8. Snell, E. E., Eakin, R. E., and Williams, R. J., *J. Am. Chem. Soc.*, **62**, 175 (1940).
9. Stokes, J. L., Larsen, A., Woodward, C. R., Jr., and Foster, J. W., *J. Biol. Chem.*, **150**, 17 (1943).
10. Hofmann, K., Melville, D. B., and du Vigneaud, V., *J. Biol. Chem.*, **141**, 207 (1941).
11. du Vigneaud, V., Dittmer, K., Hofmann, K., and Melville, D., *Proc. Soc. Exp. Biol and Med.*, **50**, 374 (1942).
12. du Vigneaud, V., Melville, D. B., Folkers, K., Wolf, D. E., Mozingo, R., Keresztesy, J. C., and Harris, S. A., *J. Biol. Chem.*, **146**, 475 (1942).
13. Melville, D. B., Dittmer, K., Brown, G. B., and du Vigneaud, V., *Science*, **98**, 497 (1943).

THE BIOLOGICAL ACTIVITY OF BIOTIN AND RELATED COMPOUNDS

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In the course of the synthesis of biotin in the Research Laboratories of Merck and Company, Inc., a number of compounds structurally related to biotin were prepared. The identity of natural and synthetic biotin was confirmed by rat and chick assays (1). The results of the bioassays, with rats, of synthetic biotin and certain biotin isomers and diamines are herein reported. These compounds have been described (2, 3). Of these preparations the following were tested on rats: biotin, *dl*-biotin, *l*-biotin, *dl*-allobiotin, diaminocarboxylic acid (corresponding to biotin), *dl*-diamino acid, *dl*-allodiamino acid, and *dl*-epiallodiamino acid (as sulfates for each diamine).

EXPERIMENTAL

Production of Biotin Deficiency—Biotin deficiency was induced in rats by feeding an egg white-containing ration of the following composition: dried egg white[1] 15, casein (Labco) 15, sucrose 52, U. S. P. Salts 1 4, dried beef liver 2, hydrogenated vegetable oil 10, cod liver oil 2, supplemented with 0.8 mg. each of thiamine and pyridoxine, 1.6 mg. of riboflavin, 10 mg. of nicotinamide, 5 mg. of calcium pantothenate, and 100 mg. of choline chloride per 100 gm.

Rats placed at weaning on this ration usually developed signs of biotin deficiency, although an occasional animal failed to become depleted. Rats maintained on this diet but supplemented with biotin remained normal in all respects. The course of biotin deficiency as reflected in the growth of rats is shown in Fig. 1. A mild degree of biotin deficiency characterized by loss of the guard hairs became apparent after about 3 weeks on the diet; at that time, the animals were still growing at a rapid rate (Period 1). In the ensuing fortnight clearly defined signs of depletion developed. The hair was sparse and mole-like. Spectacled eyes and an inflamed swollen mouth were observed; the rats had ceased to gain in weight (Period 2). During the following 2 to 3 weeks a general denudation and an exudative dermatitis with encrustations were seen. Abnormality in gait was a usual although not invariable sign; a decline in weight occurred during this stage of the deficiency (Period 3). The majority of the animals succumbed within the subsequent period of 10 days to 2 weeks. Rats in which biotin supplementation was begun during the third stage did not invariably respond to therapy; in fact many such animals gorged themselves with food and water for 3 to 4 days and died suddenly. At autopsy the stomachs were filled with food and intestinal distention was marked.

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[1] Dried egg white from Henningsen Brothers, Inc., New York.

Curative Assays—The most sensitive response to biotin was observed when supplementation was initiated during Period 2 of the depletion. Growth and healing of skin lesions were the criteria of response in the curative 14 day test. Rats were segregated into groups of like average weight and deficiency signs, negative and positive controls being employed in each assay. Negative controls were continued on the egg white diet without supplementation and positive controls received 1 γ of natural biotin[2] daily. The administration of this level of biotin produced a marked alleviation of skin symtoms; a blanching of the heretofore erythematous areas was observed in 2 to 3 days. At the conclusion of the test period the skin was normal and new hair growth was apparent.

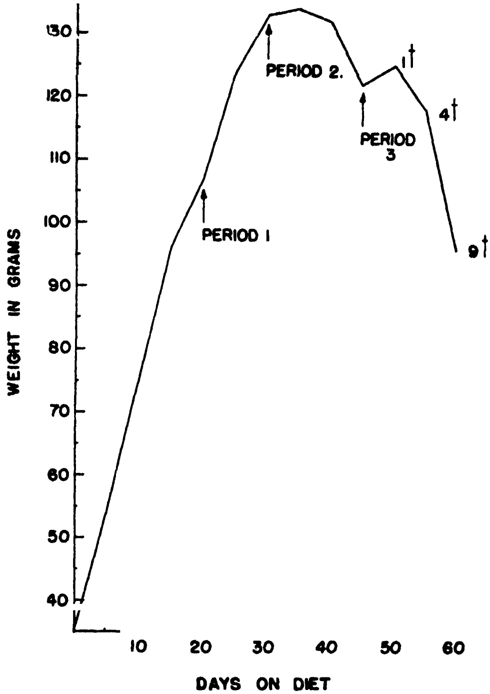

FIG. 1. Growth curve of male rats maintained on an egg white-containing diet (average of ten rats). Death of the animals is indicated by the figure followed by the dagger.

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[2] We are indebted to Dr. J. C. Keresztesy for natural biotin.

Weight gains with 1 γ of biotin were not optimal, as was evidenced by the better response to the daily administration of 2 γ of biotin. Typical weight gains for the 14 day test period were as follows: untreated controls 2 gm., 1 γ of biotin 59 gm., 2 γ of biotin 84 gm.

In the curative tests each preparation was fed daily by stomach tube for a period of 14 days. As can be seen from Table I synthetic biotin was fully utilized by the rat and *dl*-biotin was one-half as active as the naturally occurring form. The enantiomorph, *l*-biotin, was completely inactive when fed at 7½ times the level of biotin. *dl*-Allobiotin was inactive when fed at 10 times the level of biotin. These findings do not preclude a low order of potency for these compounds.

TABLE I

Biotin Activity of Biotin and Related Compounds (14 Day Curative Rat Assay)

Test No	Group	Level of compound fed	No of rats	Gain in weight, 14 days	Skin condition at conclusion of test
		γ		gm.	
1	Control		5	−12	Severe dermatitis
	Natural biotin	1	5	54	Normal
	Synthetic biotin	1	5	67	"
2	Control		5	4	Severe dermatitis
	Natural biotin	1	5	64	Normal
	dl-Biotin	2	5	60	"
	Synthetic biotin	1	5	61	"
	dl-Allobiotin	10	10	9	Severe dermatitis
3	Control		9	4	" "
	Natural biotin	1	9	53	Normal
	l-Biotin	7.5	9	−12	Severe dermatitis
4	Control		5	−5	" "
	Natural biotin	1	6	74	Normal
	Diaminocarboxylic acid corresponding to biotin (sulfate)	100*	5	−14	Severe dermatitis
	dl-Diamino acid (sulfate)	250*	5	−8	" "
	dl-Epiallodiamino acid (sulfate)	100*	5	−6	" "
	dl-Allodiamino acid (sulfate)	200*	5	−9	" "

* Equimolar equivalent of biotin.

The diamine compounds were inactive when fed at 100 to 250 times the level of biotin. It is of interest to note that the diaminocarboxylic acid derived from biotin has been reported to have 10 per cent of the activity of biotin in stimulating yeast growth (4). Its growth-promoting properties for other microorganisms are reported in an accompanying paper (5).

In rats this preparation is utilized, if at all, to an extent of less than 1 per cent.

A related compound may be active for one microorganism and not for another, as was recently demonstrated for desthiobiotin which stimulated the growth of yeast but not that of *Lactobacillus casei* (6). We have found that desthiobiotin shows a low order of activity when tested in biotin-depleted rats. However, the response was irregular and the slight activity of this compound may have been due to the presence of traces of biotin, since the compound was prepared from biotin.

SUMMARY

Synthetic and natural biotin had identical activity when assayed curatively with rats maintained on an egg white-containing ration. *dl*-Biotin was one-half as active as biotin. *l*-Biotin was inactive when fed at 7½ times the level of biotin. *dl*-Allobiotin was without effect when fed at a 10-fold increase in intake over biotin. Diaminocarboxylic acid (corresponding to biotin), *dl*-diamino acid, *dl*-allodiamino acid, and *dl*-epiallodiamino acid were inactive at levels 100 to 250 times that of biotin.

BIBLIOGRAPHY

1. Harris, S. A., Wolf, D. E., Mozingo, R., and Folkers, K., *Science*, **97**, 447 (1943).
2. Harris, S. A., Wolf, D. E., Mozingo, R., Anderson, R. C., Arth, G. E., Easton, N. R., Heyl, D., Wilson, A. N., and Folkers, K., *J. Am. Chem. Soc.*, **66**, 1756 (1944).
3. Harris, S. A., Mozingo, R., Wolf, D. E., Wilson, A. N., Arth, G. E., and Folkers, K., *J. Am. Chem. Soc.*, **66**, 1800 (1944).
4. du Vigneaud, V., Dittmer, K., Hoffman, K., and Melville, D. B., *Proc. Soc. Exp. Biol. and Med.*, **50**, 374 (1942).
5. Stokes, J. L., and Gunness, M., *J. Biol. Chem.*, **157**, 121 (1945).
6. Melville, D. B., Dittmer, K., Brown, G. B., and du Vigneaud, V., *Science*, **98**, 497 (1943).

STUDIES ON THE ACTIVITY OF SYNTHETIC BIOTIN, ITS ENANTIOMORPH, AND RELATED RACEMIC FORMS FOR THE CHICK

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(Received for publication, June 15, 1944)

Biotin from natural sources has been shown to prevent a scaly dermatitis of the feet and shanks (1, 2), to be involved in the prevention of perosis (3, 4), and to promote growth in biotin-deficient chicks (1–5). Experiments in this laboratory with chicks fed purified diets containing dried raw egg white have indicated that the above deficiency signs can be prevented by administration of synthetic biotin (6), while *l*-biotin (7, 8) and *dl*-allobiotin (7, 8) appear to be without activity in these respects.

The basal diet used in the tests contained dextrose 43.5, dried raw egg white 15, purified casein 10, cellulose 10, wheat bran 10, calcium gluconate 5, mineral mixture 3.5, glycine 1.5, wheat germ oil 0.5, fish liver oil (400 D) 0.5, *l*-cystine 0.2, choline chloride 0.15, inositol 0.1, *p*-aminobenzoic acid 0.03, niacin 0.01, calcium pantothenate 0.004, pyridoxine 0.002, riboflavin 0.002, thiamine 0.002, and menadione 0.0002 per cent.

The usual procedure has been to rear day-old white Leghorn chicks for 1 week on a commercial type of starting ration, to sort them carefully into groups of equal mean body weights and growth rates, and then feed the basal diet with or without added biotin for a 2 week period. The various biotin compounds were incorporated in aqueous solution directly into the diets, which were freshly prepared each week and fed to groups of five to seven chicks each. Electrically heated all metal battery brooders with wire floors were used throughout.

In preliminary tests it was noted that chicks receiving about 1 mg. of natural biotin[1] added per kilo of basal diet grew more rapidly than did the chicks receiving only the basal diet. The differences in weight gain were relatively large at 3 weeks of age, when the first signs of dermatitis appeared in the chicks fed the basal diet.

Comparative effects of natural and synthetic biotin upon chick growth are shown in Table I. It is seen that *dl*-allobiotin was without activity, whereas *dl*-biotin and synthetic biotin possessed activity comparable to that exhibited by the natural substance.

Activities of synthetic biotin and its enantiomorph were investigated further in an experiment in which duplicate groups of chicks were fed each level up to 30 days of age (Table II). Growth appeared to be influenced more by the presence or absence of a sufficient amount of biotin added to the diet than by the relative amount added. Severity of the dermatitis, however, was directly related to the level of synthetic biotin added to the diet. By both criteria the *l* isomer was inactive, while synthetic biotin showed complete effectiveness. The *dl*-biotin was less active than synthetic biotin, perhaps half as active, which is to be expected since the *l* isomer is inactive.

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[1] Natural biotin was supplied by Dr. J. C. Keresztesy of the Research Laboratories of Merck and Company, Inc., Rahway, New Jersey.

TABLE I

Effect of Biotin on Growth of Chicks Fed Diets Containing 15 Per Cent Dried Raw Egg White

Substance added	Experiment 1		Experiment 2		Experiment 3	
	Amount added to diet	Gain, 1-3 wks	Amount added to diet	Gain, 1-3 wks	Amount added to diet	Gain, 1-3 wks
	mg. per kg.	*gm*	*mg per kg*	*gm.*	*mg. per kg*	*gm.*
None.		56.2		67.2		45.7
Biotin (natural)	0.9	80.3	1.0	88.4	1.2	67.2
dl-Allobiotin	0.9	55.6			8.0	55.8
dl-Biotin	1.8	75.0			1.6	69.2
Biotin (synthetic)			1.0	85.2	1.2	69.3

TABLE II

Effect of Synthetic Biotin and Its Enantiomorph on Growth and Dermatitis in Chicks Fed Diets Containing 15 Per Cent Dried Raw Egg White

Substance added	Amount added to diet	Gain, 1-3 wks.	Dermatitis at 22 days*	Gain, 1-4 wks	Dermatitis at 30 days*
	mg. per kg	*gm*		*gm.*	
None..		58.6	1.2	93.0	2.7
l-Biotin..	1.6	55.9	1.4	87.6	2.9
dl-Biotin.	0.4	58.8	1.2	98.2	2.5
"	0.8	51.8	0.9	92.0	1.0
"	1.6	70.2	0.2	103.7	0.2
Biotin (synthetic)	0.4	74.4	0.8	110.2	1.4
"	0.8	69.6	0.3	106.6	0.4
"	1.6	65.8	0.3	107.2	0[illegible]

* 0.0 score was given for smooth feet and shanks showing no dermatitis; 4.0 was given for severe dermatitis; 1.0, 2.0, 3.0 were intermediate scores. Chicks of various ages reared on commercial type of diets have scored 1.0 on the scale used here.

By the end of this test perosis was present in 80 per cent of the chicks receiving no addition to the basal diet and in 20 per cent of the chicks receiving 1.6 mg. of synthetic biotin added per kilo of diet. Incidence ranged from 60 to 75 per cent on the other diets. Thus synthetic biotin also exhibits the perosis-preventing property of the natural compound.

SUMMARY

Synthetic biotin was as effective as natural biotin in promoting growth and preventing dermatitis in chicks fed a purified diet containing 15 per cent dried raw egg white. *l*-Biotin and *dl*-allobiotin were without activity.

BIBLIOGRAPHY

1. Hegsted, D. M., Mills, R. C., Briggs, G. M , Elvehjem, C. A., and Hart, E. B., *J. Nutr.*, **23**, 175 (1942).
2. Richardson, L. R., Hogan, A. G , and Karrasch, R. J., *J. Nutr.*, **24**, 65 (1942).
3. Richardson, L. R., Hogan, A. G , and Miller, O. N., *Univ. Missouri Agr. Exp. Sta., Res. Bull.* *343* (1942)
4. Jukes, T. H., and Bird, F. H , *Proc Soc. Exp Biol. and Med* , **49**, 231 (1942).
5. Waisman, H. A., Mills, R. C., and Elvehjem, C. A., *J. Nutr* , **24**, 187 (1942).
6. Harris, S. A., Wolf, D. E., Mozingo, R., and Folkers, K., *Science*, **94**, 447 (1943).
7. Harris, S. A , Wolf, D. E., Mozingo, R., Anderson, R. C., Arth, G. E., Easton, N. R., Heyl, D., Wilson, A. N., and Folkers, K., *J. Am. Chem. Soc.*, **66**, 1756 (1944).
8. Harris, S. A., Mozingo, R., Wolf, D. E., Wilson, A. N., Arth, G. E., and Folkers, K., *J. Am. Chem. Soc* , **66**, 1800 (1944).

STUDIES ON KETOSIS

XXIV. ON THE QUESTION OF BUTYRIC ACID ACTING AS A PRECURSOR OF CARBOHYDRATE*

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(Received for publication, August 24, 1944)

The fate of butyric acid has been rather extensively investigated on the rat by a number of different techniques, the results of which have not been entirely consistent. The administration of sodium butyrate by stomach tube to fasting white rats did not bring about any accumulation of glycogen in the liver (1–3), whereas the feeding of odd chain fatty acids resulted in the formation of liver glycogen. The administration of butyrate to phlorhizinized dogs led to no increase in urinary glucose (4). The excretion of acetone bodies in the urine of fasting rats given butyrate was the same as that in rats given isomolecular amounts of diacetic acid (5). These observations support the thesis that butyric acid is quantitatively oxidized and is not converted to carbohydrate.

In cat liver perfused with sodium butyrate, however, an increase in liver glycogen has been reported by Blixenkrone-Møller (6), and it was suggested that butyric acid could undergo ω oxidation to form succinic acid, a substance that has been shown to be a carbohydrate precursor (7). Stöhr (8) found that butyrate administered to rats with glucose led to a glycogen content higher than that found when glucose was given alone, and concluded that carbohydrate was formed from butyrate. Evidence that butyrate can be converted to carbohydrate has also been obtained by Buchanan, Hastings, and Nesbett (9), using butyrate in which the carboxyl group was labeled with radioactive carbon. These investigators found that radioactive carbon appeared in the liver glycogen in excess of that expected from the previously demonstrated assimilation of bicarbonate, and also in excess of that found when acetate with radioactive carbon in the carboxyl group was administered.

Very recently Dziewiatkowski and Lewis (10) found that the liver glycogen of non-fasted male white rats was higher in animals given sodium butyrate than in the controls not given this compound, suggesting either a formation of glycogen from butyrate or a sparing of glycogen by the oxidation of butyrate.

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* Some of this investigation was assisted by a grant from The Best Foods, Inc.

Since the conversion of butyrate to carbohydrate is both affirmed and denied in the foregoing investigations, we were prompted to reinvestigate the problem. It seemed possible that the nutritive state of the rats might be responsible for the discrepancies in the results. In the above investigations it was apparent that the animals in which carbohydrate formation from butyrate was not demonstrated were all fasted for 48 hours, whereas in the experiments suggesting the conversion of butyrate to carbohydrate, glucose was either simultaneously fed to fasted animals, or unfasted animals already having considerable glycogen in the liver were used. Since it is well known that the presence of carbohydrate is necessary for the proper oxidation of fatty acids with an even number of carbon atoms, it seemed possible that carbohydrate might also be essential for the conversion of such fatty acids to glycogen. Likewise, extensive fasting of the rats before butyrate administration might possibly have altered the secretion of insulin by the pancreas, as it has been shown that fasted animals have a diabetic type of glucose tolerance curve. Such an insulin insufficiency might be responsible for the failure of butyrate to be converted to glycogen by fasted rats. Accordingly, butyrate was fed to fasted rats, fasted rats previously given glucose, and to non-fasted rats, and the liver glycogen determined before and 4 hours after butyrate administration.

EXPERIMENTAL

In the first series of experiments, female rats from our stock colony were fasted 48 hours. They were divided into six groups as follows: Group 1, controls which received water by tube, to be killed after 4 hours of additional fasting; Group 2, controls which received water by tube, to be killed after 8 hours of additional fasting; Group 3, animals fed glucose, to be killed 4 hours later; Group 4, animals fed glucose, to be killed 8 hours later; Group 5, animals to be fed butyrate 4 hours later to be killed after 4 additional hours; Group 6, animals fed glucose, to be followed 4 hours later by butyrate and to be killed after 8 hours. Glucose was administered by stomach tube at a level of 400 mg. per 100 sq. cm. of surface area, as calculated by the formula of Lee (11), based on the 48 hour fasting weight. Sodium butyrate was fed as a 12 per cent solution at a level of 120 mg. per 100 sq. cm. A volume of water equivalent to that administered to the rats receiving butyrate was given to the fasted controls (Groups 1 and 2).

All rats were killed under sodium amytal anesthesia, the livers removed with the technique customarily employed in this laboratory (12), and the glycogen determined by the method of Good, Kramer, and Somogyi (13).

The gastrointestinal tract was removed, placed in a Waring blendor with 150 cc. of water, ground to a homogeneous consistency, and made up to 250 cc. after precipitation of the proteins by the addition of 10 cc. each of 0.5 N

NaOH and 10 per cent $ZnSO_4$. Reducing substances were determined on the filtrate by the Shaffer-Hartmann method and volatile acids were determined on the same filtrate by a modification of a previous method (14).

In the second series, carried out on both male and female rats which had not been fasted, the animals were divided into six groups, *viz.*, Groups 7m, 7f, killed immediately; Groups 8m, 8f, animals fed butyrate to be sacrificed after 4 hours; and Groups 9m, 9f, animals to be fasted for 4 hours and killed. In order to have data on the food consumption during the 24 hour period prior to the start of the experiment, the animals were placed in individual cages and their food consumption determined. The animals were killed and the liver glycogen determined as in the first series.

Here the homogenized gastrointestinal tract was first made to volume. An aliquot of this was hydrolyzed in 2 N HCl for 3 hours, neutralized, made to volume, and reducing substances determined. To 200 cc. of homogenized gut were added 25 cc. each of the NaOH and the $ZnSO_4$. The protein-free filtrate was used in both series for the determination of the volatile acids, as before.

The sodium butyrate was prepared by neutralization of a weighed amount of butyric acid (Eastman) to a pH of 7.4. Distillation of this preparation gave the theoretical recovery. Cerelose was used as a source of glucose, which was standardized at approximately a 50 per cent solution.

That the procedures employed in the determination of glucose and butyrate in the gastrointestinal tract were quantitative is demonstrated by the recovery of 100.6 ± 1.2 per cent of glucose (nineteen experiments) and 98.5 ± 2.0 per cent of sodium butyrate (eleven experiments) when the gastrointestinal tracts were removed immediately after their administration and the determinations made on the homogenized gut sample.

Results

The results of the first series which were carried out on fasted female rats are summarized in Table I. The most important result shown in Table I is that the liver glycogen of the groups receiving glucose (Groups 3 and 4) and glucose plus butyrate (Group 6) is identical. Also, in agreement with our previously published observations (1, 2), the administration of butyrate to fasted rats (Group 5) does not increase the liver glycogen above that of the controls (Groups 1 and 2). Virtually complete absorption of glucose and butyrate occurred before the animals were killed.

The results of studies on male and female rats, the carbohydrate stores of which had not been depleted by fasting, are shown in Table II. In this series the liver glycogen for the rats receiving butyrate is identical with that of the control group in the case of the males (Groups 8m and 9m), while a slight but statistically insignificant difference was noted with the corresponding groups of female rats (Groups 8f and 9f). In all cases there is a marked decrease in the per cent of liver glycogen following the 4 hour fasting period irrespective of whether butyrate or water was administered. The drop in liver glycogen is practically twice as great in the female rats as that obtained in the male rats. This result is in harmony with previous results which have shown sex differences in liver glycogen of fasted rats (15). The data indicate that sodium butyrate is completely absorbed and also that potential reducing material is still present in the gut after 4 hours of fasting.

TABLE I

Glycogen Formation from Glucose or Butyrate in Female Rats Previously Fasted 48 Hours

Group No	Treatment	Experimental period	No. of animals	Average body weight	Average amount fed		Reducing substances in gut as glucose*	Volatile acid in gut*	Liver	
					Glucose	Butyrate			Average weight	Glycogen*
		hrs		*gm.*	*mg*	*mg.*	*mg*	*cc. 0.1 N*	*gm.*	*per cent*
1	Fasted controls	4	8	148	0	0	15.0 ±0.1	0.84 ±0.10	4.51	<0.10
2	" "	8	11	154	0	0	10.7 ±0.1	0.78 ±0.05	4.49	<0.10
3	Glucose	4	10	144	943	0	21.4 ±5.6 (7)	1.21 ±0.12 (5)	4.95	2.42 ±0.22
4	"	8	11	147	947	0	15.8 ±2.4	0.88 ±0.80 (7)	4.71	2.48 ±0.12
5	Butyrate (fed at 4 hrs.)	8	9	144	0	299	15 4 ±0.8	1 53 ±0.24 (7)	4.49	<0.10
6	Glucose (at 0 hr.) followed by butyrate (at 4 hrs.)	8	9	148	955	302	21 5 ±5.2 (8)	1.06 ±0.10 (8)	4.71	2 18 ±0.23

When the averages are different from that of the total number of experiments, this is indicated by the figures in parentheses.

* Including the standard error of the mean calculated as follows: $\sqrt{\Sigma d^2/n}/\sqrt{n}$.

DISCUSSION

The results of this investigation do not support the contention that butyrate can be converted appreciably to liver glycogen in either fasted or fed white rats, and thus is not entirely in accord with the results of Stöhr (8) or Dziewiatkowski and Lewis (10) who, in experiments similar to those reported here, found considerably more liver glycogen in rats given butyrate than in the controls. In the first series with fasted female rats and in the second series with non-fasted males, the same liver glycogen was found in the butyrate-fed animals as in the controls. The non-fasted females in the second series do show a slight but statistically insignificant difference between the controls and those fed butyrate. It should be emphasized that in only this one instance in the four groups which received butyrate was the liver glycogen even slightly greater than in the corresponding control group. If the small difference were significant, it might be interpreted either as a conversion of a portion of the butyrate to glycogen or as a result of a sparing action of the butyrate on the metabolism of liver glycogen. At any rate, the difference would represent a conversion of only approximately 14 per cent of administered butyric acid to liver glycogen. This is in marked contrast to the results of Dziewiatkowski and Lewis (10) who found differences in liver glycogen which corresponded to over 100 per cent of the butyric acid that was fed. Possible explanations for the discrepancy between the results reported here and those of Dziewiatkowski and Lewis (10) would include the diurnal variations in the glycogen content of rat liver previously

TABLE II

Glycogen Formation from Butyrate in Unfasted Male and Female Rats

Group No *	Treatment	Experimental period	No of animals	Average body weight	Food consumed previous 24 hrs *	Total reducing substances in gut after hydrolysis (as glucose)†	Volatile acid in gut†	Liver		$\frac{\text{M.D.}}{\text{S.E M.D.}}$‡
								Average weight	Glycogen†	
		hrs		*gm*	*gm*	*mg*	*cc 0.1 N*	*gm.*	*per cent*	
7m	Controls	0	10	196	12.5 ±0.7	1090 ±104	7.46 ±0.54	8.54	4.34 ±0.24	
8m	Butyrate	4	10	195	13.6 ±0.6	403 ±38	6.37 ±0.55	7.70	3.64 ±0.24	
9m	Fasted controls	4	10	198	14.5 ±0.5	453 ±46	6.45 ±0.53	8.06	3.57 ±0.39	0.19
7f	Controls	0	18	159	10.5 ±0.5 (9)	392 ±46 (9)	4.30 ±0.36 (9)	6.40	4.06 ±0.13	
8f	Butyrate	4	19	154	9.0 ±0.6 (10)	238 ±18 (10)	4.18 ±0.35 (10)	5.92	2.77 ±0.21	
9f	Fasted controls	4	20	161	13.0 ±3.7 (10)	262 ±26 (10)	3.40 ±0.39 (10)	5.89	2.07 ±0.21	2.34

When the averages are different from that of the total number of experiments, this is indicated by the figures in parentheses.

* Male and female rats are indicated by the letters.

† Including the standard error of the mean calculated as follows: $\sqrt{\Sigma d^2/n}/\sqrt{n}$.

‡ Mean difference to standard error of mean difference between Groups 8 and 9. When this ratio exceeds 3.0, the results are considered significant.

described (12). Such diurnal changes make it necessary to run simultaneous control and experimental animals in order adequately to minimize liver glycogen differences not due to the experimental treatment.

The experiments of Buchanan, Hastings, and Nesbett (9) indicate that more radioactive carbon appeared in liver glycogen after radiopropionate or radiobutyrate than after radioacetate administration, the tagged carbon atom being in the carboxyl groups in all cases. When the radioactive carbon content of the liver glycogen was corrected for that expected from the assimilation of radiobicarbonate, it appeared that butyric and propionic acids were converted to glycogen, whereas acetic acid was not. The conversion of butyrate (and of propionate) to glycogen indicated by the above experiments amounted to less than 5 per cent of the fatty acid absorbed, which corresponded to about 3 mg. of glycogen. This would not constitute a demonstrable synthesis under the conditions of our experiments.

Although the possibility of some conversion of butyric acid to liver glycogen cannot be eliminated on the basis of the present work, it is clear that this occurs at most only to a very limited extent.

SUMMARY

The administration of sodium butyrate to fasting female rats fed glucose 4 hours earlier did not augment the liver glycogen over that of control animals. Also, the feeding of this substance to unfasted male and female rats did not lead to statistically significant differences over those of the controls.

BIBLIOGRAPHY

1. Deuel, H. J., Jr., Butts, J. S., Hallman, L. F., and Cutler, C. H., *J. Biol. Chem.*, **112**, 15 (1935).
2. Butts, J. S., Blunden, H., Goodwin, W., and Deuel, H. J., Jr., *J. Biol. Chem.*, **117**, 131 (1937).
3. Deuel, H. J., Jr., Butts, J. S., Blunden, H., Cutler, C. H., and Knott, L., *J. Biol. Chem.*, **117**, 119 (1937).
4. Ringer, A. I., *J. Biol. Chem.*, **14**, 43 (1913).
5. Butts, J. S., Cutler, C. H., Hallman, L., and Deuel, H. J., Jr., *J. Biol. Chem.*, **109**, 597 (1935).
6. Blixenkrone-Møller, N., *Z. physiol. Chem.*, **252**, 137 (1938).
7. Ringer, A. I., Frankel, E. M., and Jonas, L., *J. Biol. Chem.*, **14**, 539 (1913).
8. Stöhr, R., *Z. physiol. Chem.*, **217**, 141 (1933).
9. Buchanan, J. M., Hastings, A. B., and Nesbett, F. B., *J. Biol. Chem.*, **150**, 413 (1943).
10. Dziewiatkowski, D. D., and Lewis, H. B., *J. Biol. Chem.*, **153**, 49 (1944).
11. Lee, M. O., *Am. J. Physiol.*, **89**, 24 (1929).
12. Deuel, H. J., Jr., Butts, J. S., Hallman, L. F., Murray, S., and Blunden, H., *J. Biol. Chem.*, **123**, 257 (1938).
13. Good, C. A., Kramer, H., and Somogyi, M., *J. Biol. Chem* , **100**, 485 (1933).
14. Deuel, H. J., Jr., Hallman, L., and Reifman, A., *J. Nutr.*, **21**, 373 (1941).
15. Butts, J. S., and Deuel, H. J., Jr., *J. Biol. Chem.*, **100**, 415 (1933).

TWO MICROBIOLOGICAL METHODS FOR THE DETERMINATION OF *l*(−)-TRYPTOPHANE IN PROTEINS AND OTHER COMPLEX SUBSTANCES

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While microbiological assays for the determination of nicotinic acid by the method of Snell and Wright (1) were being made, it was observed that *Lactobacillus arabinosus* 17-5 would not grow unless the medium contained tryptophane. The addition of 4 γ to 40 γ of *l*(−)-tryptophane to 10 ml. of the medium resulted in increasing growth and production of acid. An attempt, therefore, was made to develop an assay method utilizing this organism.[1]

The different chemical methods of determining tryptophane in pure proteins have yielded varying results in the hands of careful workers. The amount of tryptophane in casein, when determined by the different chemical methods, has been reported to be from 1.0 per cent to 2.4 per cent (7–10). (A critical review of these methods has been made by Horn and Jones.[2]) It is generally known that interfering colors develop when attempts are made to assay tryptophane in materials that contain carbohydrates or other color-producing substances, thereby rendering the test impractical for tryptophane determinations in such materials. Also tryptophane cannot be determined in materials containing small amounts of this amino acid.

Two methods of assay of *l*(−)-tryptophane with bacteria were developed. In one the acid produced in cultures of *Lactobacillus arabinosus* 17-5 is titrated and in the other the turbidity caused by the growth of *Eberthella typhosa* T-63 is read in a photometer. Each of the methods has the following advantages over the chemical assay methods commonly employed.

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[1] Since this work was started, reports have appeared on the use of *Lactobacillus arabinosus* or of *Lactobacillus casei* for the analysis of eleven amino acids in pure amino acid combinations (2, 3); for the assay of valine, leucine, and isoleucine in cottonseed meal, peanut meal, soy bean meal, wheat, and gelatin (4), of valine and arginine in protein hydrolysates (5), and of tryptophane in casein (6).

[2] Dr. Millard J. Horn and Dr. D. Breese Jones, of the Bureau of Human Nutrition and Home Economics of the United States Department of Agriculture, are reporting a chemical assay method for tryptophane. They have collaborated with us by making nitrogen and moisture determinations and assays for tryptophane by their method, on certain hydrolysates that were assayed by our microbiological method. Their report appears in the following paper.

(1) Minute amounts of tryptophane in materials for assay can be determined. (2) Substances that interfere with the determination of tryptophane by the chemical methods do not affect the microbiological assays. (3) The microbiological tests can be performed by personnel with little technical training. (It must be remembered that *Eberthella typhosa* is a pathogen and should be used with caution, not only to protect the health of the personnel but to prevent the contamination of foods being assayed. *Lactobacillus arabinosus* is non-pathogenic and therefore can be handled with impunity.)

The microbiological assay with *Eberthella typhosa* has the advantage over the *Lactobacillus arabinosus* method in that readings can be made after only 16 hours of incubation, no titration is required since readings are made directly on the incubation tubes, and the medium is simpler, being prepared from a mixture of pure amino acids. The method, therefore, may be applicable to the assay of other amino acids. Although the *L. arabinosus* assay also can be carried out with media containing pure amino acids, *L. arabinosus* requires larger amounts of amino acids for maximum growth, and the synthetic media are therefore more expensive to prepare. The principal disadvantage is the danger to personnel working with *Eberthella typhosa;* for this reason most laboratories will probably prefer to use the *L. arabinosus* assay method.

Lactobacillus arabinosus Assay Method

Preparation of Medium—A modification of the medium used in the assay of nicotinic acid (1) is employed. The modifications are as follows: (1) casein is hydrolyzed with hydrochloric acid and the excess acid is removed by successive evaporation to a thick syrup three times over a steam bath in a hood; (2) the treatment with activated charcoal is carried out without adjusting the pH; and (3) tryptophane is left out of the medium and nicotinic acid is added.

The tryptophane-free medium for 100 assay tubes consists of hydrolyzed casein (10 per cent) 100 ml., dextrose 25 gm., sodium acetate 20 gm., cystine 100 mg., adenine sulfate 10 mg., guanine hydrochloride 10 mg., uracil 10 mg., inorganic Salt Solutions A and B 5 ml. each, thiamine hydrochloride 200 γ, pyridoxine hydrochloride 200 γ, *p*-aminobenzoic acid 200 γ, calcium pantothenate 400 γ, nicotinic acid 400 γ, riboflavin 400 γ, biotin 1 γ; adjust to pH 6.6 to 6.8 with NaOH, distilled water to make 500 ml. Solutions of adenine, guanine, and uracil, of inorganic Salts A and B and of the various vitamins are prepared separately. It is important that the solution of calcium pantothenate is prepared fresh at least every 2 weeks.

Procedure—Assays are carried out in large test-tubes, approximately 22 mm. in diameter. The standard l(−)-tryptophane solution and solutions for analysis are added to the tubes. Duplicate tubes containing 0, 4, 8, 12, 16, 20, 24, 28, 32, 36, and 40 γ of *l*(—)-tryptophane are set up for the standard curve (Fig. 1). Samples for assay are set up at increasing levels of concentration estimated to approximate the amounts of tryptophane contained in the tubes for the standard curve. Volumes are brought to 10 ml. with distilled water and 5 ml. of the prepared medium are added. The tubes are plugged with cotton and sterilized by being autoclaved for 15 minutes at 15 pounds pressure. Each of the tubes except the first is inoculated as in nicotinic acid assays (1). Incubation is carried out at 37° for 3 days and acid production is titrated with 0.1 N NaOH to neutrality.

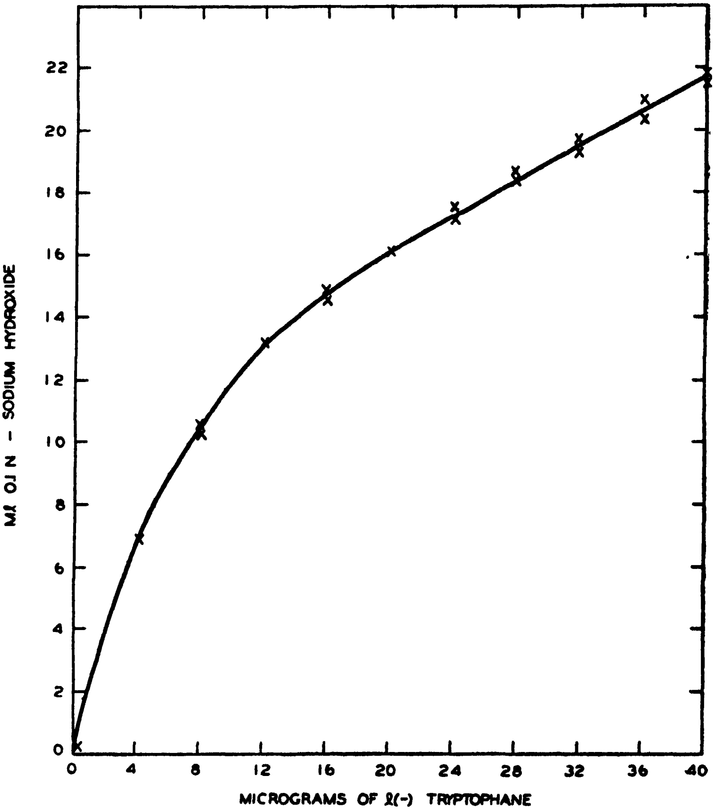

FIG. 1. A typical *l*(—)-tryptophane standard curve with *Lactobacillus arabinosus*

Eberthella typhosa Assay Method

Fildes (11) observed that *Bacterium typhosum* ordinarily will not grow unless tryptophane is present in the nutrients, but that it could be trained to grow without tryptophane which is then synthesized. Burrows (12) showed that a strain of *Bacteria typhosum*, which required tryptophane to start growth, synthesized not only tryptophane but also indole. We were fortunate to obtain a strain of *Eberthella typhosa*, National Institute of Health No. T-63, that required tryptophane but did not have the capacity of synthesizing tryptophane or indole in 3 to 5 days when started with less than optimum amounts of either of the compounds. It was, therefore, decided to utilize this organism for a microbiological assay method.

Preparation of Medium—The basal medium for the assay method in which *Eberthella typhosa* T-63 is used is a slight modification of the amino acid medium described by Gladstone (13).

The following stock solutions are prepared.

Solution A—*dl*-Alanine 1.19 gm., *dl*-valine 1.56 gm., *dl*-leucine 1.75 gm., *dl*-glycine 1.0 gm., *l*-proline 0.77 gm., *l*-hydroxyproline 1.09 gm., *dl*-aspartic acid 1.77 gm., *d*-glutamic acid 0.98 gm., *dl*-methionine 0.75 gm., *dl*-phenylalanine 0.82 gm., *l*-tyrosine 0.45 gm., *l*-arginine monohydrochloride 0.52 gm., *l*-histidine monohydrochloride 0.5 gm., *l*-lysine 0.46 gm., KH_2PO_4 45.3 gm., N NaOH 260.0 ml. The amino acids are dissolved by heat if necessary, then cooled, and distilled water sufficient to make 2500 ml. is added.

Solution B—*l*-Cystine 1.0 gm., hydrochloric acid 5.0 ml. Water sufficient to make 333.0 ml. is added. The cystine is then dissolved by heat. The solution is then cooled to room temperature and is brought to volume with water.

Solution C—$MgSO_4 \cdot 7H_2O$, 0.36 gm., is dissolved in 100 ml. of water.

All solutions are kept under toluene.

To prepare medium for 100 assay tubes, 20 gm. of dextrose are dissolved in water and 250 ml. of Solution A, 20 ml. of Solution B, and 10 ml. of Solution C are added. The pH is adjusted to 7.6 and the volume is brought to 500 ml. with water.

Procedure—The assays are carried out in 22 × 175 mm. precision tubes standardized for use in an Evelyn photometer. Standard and assay solutions are prepared as in the previous method. Duplicate tubes containing 0, 0, 4, 8, 12, 16, and 20 γ of *l*(−)-tryptophane are set up for the standard curve (Fig. 2). Samples for assay are set up at increasing levels of concentration estimated to approximate the amounts of tryptophane contained in the tubes for the standard curves. The volume is brought to 5 ml. with distilled water, and 5 ml. of the medium are added. The tubes are plugged with cotton and sterilized by autoclaving for 15 minutes at 15 pounds pressure. The inoculum is prepared by transferring the organisms from a recent agar slant culture to nutrient broth and incubating for from 18 to 20 hours. The cultures are centrifuged and the supernatant liquid is poured off. The residue is brought back to volume with 0.85 per cent sodium chloride solution and mixed. 1 ml. of the suspended organisms is added to 19 ml. of 0.85 per cent sodium chloride solution. Each of the tubes, except the first two, which are used as balancing tubes for the photometer, is inoculated with approximately 0.05 ml. from a 1 ml. pipette of the dilute suspension of the organisms. The tubes are incubated for 16 hours at 37°, and the turbidity is read with a photometer.

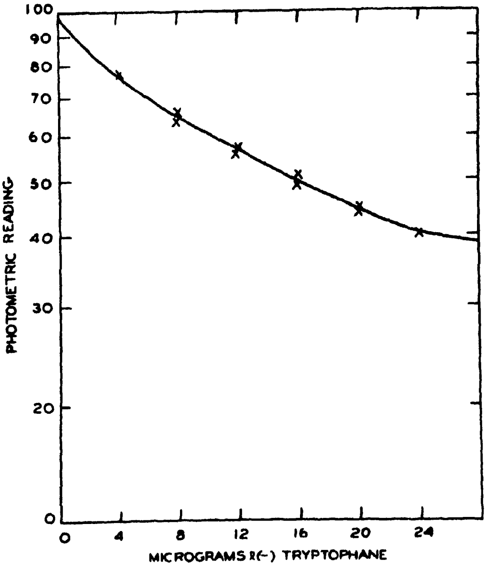

Fig 2 A typical *l*(−)-tryptophane standard curve with *Eberthella typhosa*

Specificity of Methods—Data on the growth-promoting activity of a number of substances for the two organisms as compared to *l*(−)-tryptophane are given in Table I. One sample of *dl*-tryptophane had 65 per cent of the activity of *l*(−)-tryptophane. We were not able to explain this increased activity, nor were the manufacturers. Indole is the only material so far tested that is of importance in interfering with the test. It can be readily removed from an aqueous solution without removal of any of the tryptophane by shaking with ether or toluene. However, tests for indole were made with Böhme's reagent (14) on twenty-eight samples that had been hydrolyzed with 5 N NaOH for from 6 to 40 hours in an autoclave at 15 pounds pressure, and stored under toluene. These samples consisted of *l*(−)-tryptophane, casein, beef, pork, veal, lamb, wheat, peas, and potatoes. Of these only *l*(−)-tryptophane that had been autoclaved for 40 hours gave a positive reaction. In this hydrolysate, a solution of 1 mg. of tryptophane contained 0.1 γ of indole. Solutions of arachin and of ox muscle protein that had been digested with pepsin, trypsin, and erepsin, and of casein, lactalbumin, peanut meal, cottonseed meal, soy bean meal, corn germ, wheat germ, black-eyed peas, and wheat that had been digested with pepsin and trypsin each gave negative tests for indole. This test for indole, in our hands, gave a positive reaction when 0.1 γ of indole was present in 10 ml. of aqueous suspension. From these observations it is believed that the interference from indole can be disregarded and that the growth of the two organisms, under the conditions employed, is limited by the amount of *l*(−)-tryptophane that is added to the media.

TABLE I

Activity of Pure Substances for Lactobacillus arabinosus 17-5 and Eberthella typhosa T-63 as Compared to l(−)-Tryptophane

Substance	*Lactobacillus arabinosus*	*Eberthella typhosa*
	per cent	*per cent*
Indole	90–120 (Molecular)	180–240 (Weight)
dl-Tryptophane Sample 1*	65	64
" " 2*	50	50
" " 3†	50	50
3-Indolealdehyde*	0	0
3-Indolal hydantoin*	0	0
" thiohydantoin*	0	0
γ-(Indole-3)-*n*-butyric acid	0‡	0
Indole-3-acetic acid	0‡	0.07
β-(Indole-3)-propionic acid	0‡	0

* Obtained through the courtesy of Dr L. E. Livak, The Dow Chemical Company.

† Merck and Company, Inc.

‡ Reported by Greene and Black (6).

Comparisons of the Two Methods—Comparison of the determination of *l*(−)-tryptophane in hydrolysates by the two microbiological methods (Table II) indicates that essentially identical results are obtained.

The results discussed in the remainder of this report are based on assays with *Lactobacillus arabinosus*.

Preparation of Materials for Assay—Sodium hydroxide treatments and enzymatic digestion for the liberation of tryptophane were investigated. Greene and Black (6) stated that tryptophane has been considered stable in sodium or barium hydroxide, though subject to racemization. They studied the effect of barium hydroxide digestion of casein and found that casein, when treated with 20 volumes of 5 N $Ba(OH)_2$ and autoclaved for about 5 hours at 15 pounds pressure, yielded assay values of *l*(−)-tryptophane equal to about one-half the values obtained from pancreatic digestion. They assumed that complete racemization was effected during alkaline hydrolysis. We investigated the effect of different methods of sodium hydroxide hydrolyses on the assay values of $l(-)$-tryptophane in different materials. 50 ml. of 5 N NaOH were added to each gm. of $l(-)$-tryptophane, or pure protein, and to each estimated gm. of protein in more complex substances. Different samples of each were autoclaved at 15 pounds pressure for different lengths of time, refluxed in an oil bath at 175° for 6 hours, or refluxed for 6 hours over an electric hot-plate. Pure $l(-)$-tryptophane assayed 92 per cent of its original activity after being refluxed 6 hours over a hot-plate, 74 per cent was found after being autoclaved 6 hours, and 68 per cent after being autoclaved 40 hours, respectively, with

TABLE II

Results of Assays for $l(-)$-Tryptophane by Lactobacillus arabinosus 17-5 and Eberthella typhosa T-63

Sample	Method of hydrolysis*		Per cent of $l(-)$-tryptophane found	
			Lactobacillus arabinosus	*Eberthella typhosa*
		hrs.		
$l(-)$-Tryptophane	Autoclaved†	6	74	77
“	“	40	68	67
“	Refluxed‡	6	92	96
Casein	“	6	0.45	0.50
“	“ §	6	0.50	0.53
“	Autoclaved	6	0.60	0.60
“	“	20	0.46	0.51
“	“	40	0.53	0.53
Arachin	Refluxed‡	6	0.35	0.35
Conarchin	“	6	0.43	0.44
Glycinin	“	6	0.41	0.42
Wheat bran albumin	“	6	1.25	1.24
Lactalbumin	“	6	0.73	0.73
Black-eyed peas	Autoclaved	6	0.10	0.10
“ “	“	40	0.135	0.135
Wheat	“	6	0.056	0.055
“	“	40	0.076	0.074
Horse meat	“	40	0.12	0.113
Smoked ham	Refluxed§	6	0.10	0.10

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* Hydrolyzed in 50 ml. of 5 N NaOH for each gm. of $l(-)$-tryptophane or for each estimated gm. of protein.

† Autoclaved at 15 pounds pressure.

‡ Refluxed over an electric hot-plate.

§ Refluxed in an oil bath at 175°.

the alkali. Several samples of casein were similarly treated. The assay values were 0.42 to 0.5 per cent after the samples were refluxed for 6 hours over a hot-plate, 0.53 to 0.71 per cent after being refluxed in an oil bath 6 hours, 0.46 to 0.51 per cent after being autoclaved 20 hours, and 0.46 to 0.53 per cent after 40 hours in the autoclave. The values for black-eyed peas were 0.10 per cent after 6 hours and 0.135 per cent after 40 hours in the autoclave, and for wheat 0.056 and 0.076 per cent after similar treatments. Values of 1.18 per cent for casein, 0.21 per cent for black-eyed peas, and 0.11 per cent for wheat, after enzymatic digestion, were obtained. These results indicate that racemization of pure $l(-)$-tryptophane takes place slowly when the substance is refluxed or autoclaved with 50 volumes of 5 N NaOH; that irregular values are obtained in casein after exposure to the alkali under the same conditions; and that digestion of peas and wheat takes place slowly.

Enzymatic digestions were next studied. The effect of pepsin, trypsin, papain, taka-diastase, polidase, pangestin, erepsin, chymotrypsin and chymotrypsinogen,[3] either individually or in various combinations, was tested. The enzyme combinations that gave the highest yield of $l(-)$-tryptophane in casein, *viz.* 1.36 per cent and 1.35 per cent were (1) pepsin, trypsin, and chymotrypsin, and (2) pepsin, trypsin, and chymotrypsinogen. Unfortunately, we were able to obtain only a sufficient amount of two of the enzymes (chymotrypsin and chymotrypsinogen) to digest two small samples of casein with each. The combinations of commercially obtainable enzymes that gave the highest repeatable yields of tryptophane in pure proteins, corn and wheat germs, flours, black-eyed peas, and wheat were pepsin, trypsin, and erepsin, or pepsin and trypsin.

Procedure for Digesting Materials with Pepsin, Trypsin, and Erepsin, and with Pepsin and Trypsin—The amount of assay material estimated to contain 1.0 gm. of protein is weighed into a 100 ml. Erlenmeyer flask; 50 ml. of 0.1 N H_2SO_4, and 10 mg. of pepsin (1:10,000 potency) are added. The flask is incubated overnight at 37°, then 3.0 gm. of $K_2HPO_4 \cdot 12H_2O$ are added, and the pH is adjusted to 8.4. 10 mg. of trypsin (1:300 potency) are added and the flask is again incubated 12 to 24 hours at 40°. After the pH is adjusted to 7.8, 100 mg. of erepsin are added and the flask is incubated for 2 days at 40°. The pH is then adjusted to 6.8 and the volume is brought to 100 ml. with water. The flask is shaken occasionally during each incubation period. The contents are kept under toluene throughout the process of digestion.

A similar amount of material to be assayed is treated with pepsin as above. After incubation, the basic phosphate is added and the pH is adjusted to 8.4. 50 mg. of trypsin are added and the flask is incubated for 3 days at 40°. The pH is adjusted to 6.8 and the volume is brought to 100 ml.

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[3] Received through the courtesy of Dr. Dean Burk of the National Cancer Institute of the National Institute of Health.

Enzyme controls are digested with each procedure, the difference being that 10 times the amount of enzymes employed in digesting the assay materials are digested without the addition of the assay materials. The amount of tryptophane calculated to be present in the enzymes used in the digestion is deducted from the total amounts of tryptophane found in the assayed materials.

TABLE III

Results of l(−)-Tryptophane Recovery When Added to Materials and Hydrolyzed with Sodium Hydroxide and When Digested with Pepsin and Trypsin

Material	Method of hydrolysis	Per cent of added *l*(−)-tryptophane recovered
Casein	Refluxed over hot-plate 6 hrs with NaOH	80
"	Autoclaved 20 hrs. with NaOH	96
"	" 40 " " "	94
Black-eyed peas	Refluxed over oil bath 6 hrs with NaOH	77
" "	Autoclaved 6 hrs. with NaOH	92
" "	" 40 " " "	88.5
Whole wheat	" 6 " " "	83
" "	" 40 " " "	83
Casein	Pepsin and trypsin digestion	100
Lactalbumin	" " " "	102
Cottonseed flour	" " " "	99
Soy bean flour	" " " "	102
Corn germ	" " " "	101

In actual assays, evidence of complete digestion of the materials is indicated only when there is uniform comparison of the assay material and the *l*(−)-tryptophane standard throughout. If digestion is incomplete, ascending values result when increased amounts of the assay material are added to the culture tubes.

As a further test of the two methods of digestion, known amounts of *l*(−)-tryptophane were added to the materials before hydrolysis and the per cent of recovery of this tryptophane was determined.

The recovery of *l*(−)-tryptophane after sodium hydroxide hydrolysis with assay materials gave irregular results. The recovery varied between 77 and 96 per cent. When *l*(−)-tryptophane was added to materials and digested by pepsin and trypsin, the recovery was from 99 to 102 per cent (Table III). This adds further evidence that alkali hydrolysis is unsatisfactory as a method of preparing materials for the assay of tryptophane by the microbiological method.

Assays by Microbiological and Chemical Methods—Samples of proteins and other materials that had been hydrolyzed with sodium hydroxide and that had been digested by pepsin, trypsin, and erepsin were assayed independently by Horn and Jones by their chemical method and by us by our microbiological method (Table IV).

TABLE IV

Nitrogen and Tryptophane Values for Proteins and Other Substances on Moisture-Free Basis Expressed in Per Cent

Material	Nitrogen*	Digested by pepsin, trypsin, and erepsin		Refluxed with sodium hydroxide over electric hot-plate	
		Microbiological method	Chemical method*	Microbiological method	Chemical method*
Labco casein	14.42	1.20	1.20	0.47	1.10
Arachin	18.30	1.08	0.97	0.38	0.70
Lactalbumin	15.40	1.79	1.79	0.75	1.74
Ox muscle protein	16.00	1.17	1.18	0.45	1.24
Phaseolin	16.10	0.38	0.38	0.25	0.70
Lima bean globulin	15.10	0.90	0.89	0.60	1.01
Velvet bean globulin	16.70	0.73	0.73	0.34	0.69
Cottonseed flour (Proflo)	9.88	0.63	0.64	0.36	0.89
Peanut flour	9.85	0.55	0.56	0.28	0.59
Soy bean flour	8.81	0.55	0.62	0.36	0.87
Wheat germ (defatted)	6.58	0.36	0.33	0.33	0.40
Corn " "	3.74	0.24	0.26	0.22	0.33
Black-eyed peas	4.21	0.24	0.22	0.15†	
Whole wheat	2.14	0.13	0.14	0.09†	

* Determined by Horn and Jones (foot-note 2).

† Autoclaved, 40 hours with 50 ml. of 5 N NaOH per estimated gm. of protein.

When samples were hydrolyzed with sodium hydroxide and assayed by both methods, wide differences were obtained. The values of seven proteins assayed by the microbiological method gave from 25 to 60 per cent of the values obtained by the chemical method. The values of three flours were from 40 to 49 per cent and of corn and wheat germs 67 and 82 per cent, respectively, of those shown by the chemical method. The high values obtained by microbiological assay of the germs, peas, and wheat as compared to those obtained by the chemical method on these hydrolysates, or to those obtained by the microbiological assay of these substances after digestion with enzymes, could be explained only by probable incomplete racemization by the sodium hydroxide hydrolysis of the *l*(−)-tryptophane contained in these materials.

The results of the assays of the enzyme digests by the two methods were in very close agreement.

SUMMARY

1. Two methods of assaying $l(-)$-tryptophane, one by utilizing *Lactobacillus arabinosus* 17-5, and the other *Eberthella typhosa* T-63, are described.

2. The assay of $l(-)$-tryptophane by the two methods gave identical results.

3. Irregular $l(-)$-tryptophane values are obtained in sodium hydroxide hydrolysates of proteins and in protein-containing foodstuffs.

4. The digestion of proteins and other materials by enzymes and the superiority of this form of hydrolysis for microbiological assays are discussed.

5. Close agreement in the amount of tryptophane in materials digested by enzymes is obtained in assays both by the chemical method of Horn and Jones and by our microbiological method.

We wish to express our appreciation to Mrs. C. M. Palmes for her technical assistance with some of the enzyme digestions and assays.

BIBLIOGRAPHY

1. Snell, E. E , and Wright, L. D., *J. Biol. Chem.*, **139**, 675 (1941).
2. Shankman, S., Dunn, M. S., and Rubin, L. D., *J. Biol. Chem.*, **150**, 477 (1943).
3. Shankman, S., Dunn, M. S., and Rubin, L. D., *J. Biol. Chem.*, **151**, 511 (1943).
4. Kuiken, K. A., Norman, W. H., Lyman, C. M., Hale, F., and Blotter, L., *J. Biol. Chem.*, **151**, 615 (1943).
5. McMahan, J. R., and Snell, E. E., *J. Biol Chem.*, **152**, 83 (1944).
6. Greene, R. D., and Black, A., *Proc. Soc. Exp. Biol. and Med.*, **54**, 322 (1943).
7. May, C. E., and Rose, E. R , *J. Biol. Chem.*, **54**, 213 (1922).
8. Sullivan, M. X., Milone, H. S., and Everitt, E. L., *J. Biol. Chem.*, **125**, 471 (1938).
9. Shaw, J. L. D., and McFarlane, W. D., *Canad. J. Res.*, *Sect. B*, **16**, 361 (1938).
10. Block, R. J , and Bolling, D., The determination of amino acids, Minneapolis, revised edition (1940).
11. Fildes, P., *Brit. J. Exp. Path.*, **21**, 21 (1940).
12. Burrows, W., *J. Infect. Dis.*, **65**, 134 (1939).
13. Gladstone, G. P., *Brit. J. Exp. Path.*, **18**, 67 (1937).
14. Topley, W. W. C., and Wilson, G. S., The principles of bacteriology and immunity, Baltimore, 2nd edition, p. 258 (1937).

A RAPID COLORIMETRIC METHOD FOR THE DETERMINATION OF TRYPTOPHANE IN PROTEINS AND FOODS*

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A survey of the literature shows that the values given by several investigators for the tryptophane content of certain proteins are twice those reported by others for the same protein. In practically all cases the high values were obtained by colorimetric methods in which the color developed was compared against that similarly obtained with casein, the value for the casein having been predetermined by the same procedure with free tryptophane as a standard. The tryptophane values for casein thus found ranged from 2 to 2.4 per cent. These high values for tryptophane in casein have been reported by May and Rose (1) and others (2–5) who used their method or modifications of it. On the other hand, Shaw and McFarlane (6), Block and Bolling (7), with different colorimetric procedures, and Greene and Black (8), by a microbiological method, found casein to contain from 1 to 1.20 per cent of tryptophane.

Sullivan, Milone, and Everitt (4) developed a modification of the May and Rose method which greatly shortened the time required for the determination of tryptophane. When casein was heated with hydrochloric acid and *p*-dimethylaminobenzaldehyde at 85° for 15 minutes, a blue color developed after the addition of 0.3 per cent hydrogen peroxide, which remained permanent for 24 hours. The values for casein were identical with those obtained after 8 days digestion according to the May and Rose method. The color thus obtained was then compared with that given by a standard solution of free tryptophane, treated according to the May and Rose method. They found 2.4 per cent tryptophane for casein.

Shaw and McFarlane (9) made a critical study both of the May and Rose method and the rapid method of Sullivan, Milone, and Everitt, and showed that erroneous results are obtained by these methods, due to the fact that tryptophane, as combined in the protein molecule, gives more color with the *p*-dimethylaminobenzaldehyde reagent than does an equivalent amount of free tryptophane under the same conditions. They also showed that the mode of linkage and degree of oxidation of tryptophane influence the color reaction, so that the source of error in the procedure lies in the use of free tryptophane as a standard.

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* The determination of tryptophane in proteins and foodstuffs was begun as a collaborative study with Dr. Jerald G. Wooley and Dr. W. H. Sebrell of the National Institute of Health, who are reporting the determination of tryptophane by microbiological methods in the preceding paper.

We have confirmed the findings of Shaw and McFarlane (9) and found that free tryptophane determined by the rapid method of Sullivan, Milone, and Everitt, or by the long May and Rose method, gives only one-half the maximum color obtained by an equivalent amount of tryptophane when combined in the casein molecule. However, the several methods for determining tryptophane, in which *p*-dimethylaminobenzaldehyde has been employed, can be used with consistent results only if it is kept in mind that when the tryptophane is in peptide union the maximum color develops either at 85° or at room temperature, but when the tryptophane is free the maximum color develops only at room temperature. By use of the method here described the tryptophane content of casein was found to be 1.1 to 1.2 per cent, and the values for other proteins approximately one-half of the higher values that have been reported.

The excellent method of Shaw and McFarlane, as applied to purified proteins, is inapplicable to hydrolysates of materials containing carbohydrates such as meals and flours, because of darkening of the solution by the sulfuric acid used. Although concentrated hydrochloric acid as used in the method of May and Rose does not produce the darkening effect, it has, however, the disadvantage of requiring several days for development of the maximum color. Furthermore, so long a contact with a reactive agent such as *p*-dimethylaminobenzaldehyde may involve secondary reactions that may interfere with measurement of true tryptophane values.

Because of the destructive effect of acid hydrolysis on tryptophane in proteins, sodium hydroxide is generally used as the hydrolytic agent. Although satisfactory when used with isolated proteins, alkaline hydrolysis cannot be applied to material such as meals, flours, and whole seeds because the carbohydrates present produce colored solutions which interfere with colorimetric determinations. This difficulty has been satisfactorily met by overnight digestion of the material with papain. After removal of the fiber and most of the starch, light colored solutions of the protein material are obtained.

Tryptophane determinations made on a number of isolated proteins and foodstuffs by the procedure herein described gave results closely agreeing with those obtained independently on the same materials by the microbiological methods of Wooley and Sebrell.

EXPERIMENTAL

Determinations of Free Tryptophane—That the maximum color for free tryptophane is not developed by the short method of Sullivan, Milone, and Everitt at 85° was shown by the following series of experiments.

10 mg. of pure tryptophane were dissolved in water and the solution was made up to 100 cc. A 0.5 cc. aliquot was introduced into an Evelyn colorimeter tube, followed by 0.5 cc. of a 5 per cent solution of *p*-dimethylaminobenzaldehyde in concentrated hydrochloric acid. Finally, there were added 1 cc. of water and 5 cc. of concentrated hydrochloric acid. A pink color developed almost immediately. After the solution had stood for 5 minutes at room temperature, during which time the pink color had faded somewhat, 1 drop of 0.2 per cent sodium nitrite solution was added. Within a few seconds a pure blue color developed. A tube containing 2 cc. of water, 5 cc. of concentrated hydrochloric acid, and 1 drop of sodium nitrite solution was used as a blank for color comparison. After standing for 2 minutes, a color reading of 82 was observed which remained constant for 1 hour.

A second aliquot of the tryptophane solution was treated in the same manner as the first, with the exception that 1 drop of a 0.3 per cent solution of hydrogen peroxide was added instead of the sodium nitrite. The tube was placed in the colorimeter immediately. The blue color gradually developed to a maximum reading of 82. This value remained constant for about 30 seconds, after which it gradually faded.

A third tube containing the same reagents except the oxidizing reagent was heated for 15 minutes at 85° and a drop of hydrogen peroxide was added. After the tube was cooled, a reading of 89.5 was observed. This value represents a color intensity approximately one-half of that obtained with aliquots treated at room temperature.

In order to develop data for plotting standard curves for free tryptophane values, colorimetric measurements by the three different methods described above were carried out with aliquots containing 0.0, 0.02, 0.04, 0.06, and 0.08 mg. of tryptophane. The maximum readings obtained with hydrogen peroxide and sodium nitrite, after the material stood at room temperature for 5 minutes, were in every case twice those obtained with hydrogen peroxide at 85°, and the results are shown in Table I. The reading, 92, for 0.04 mg. concentration obtained at 85° is approximately the same as the reading, 91.5 obtained for 0.02 mg. concentration at room temperature.

Standard Curves for Free Tryptophane—Standard tryptophane curves were prepared by using eleven colorimetric tubes containing from 0.0 cc. to 1 cc. (ranging in increasing order of 0.1 cc.) of an aqueous tryptophane solution containing 10 mg. per 100 cc.; and 0.5 cc. of a 5 per cent solution of *p*-dimethylaminobenzaldehyde in concentrated hydrochloric acid was added to all of the tubes except the blank. The eleven tubes were then made up to 2 cc. with distilled water, and 5 cc. of concentrated hydrochloric acid were added to each tube and the mixture shaken. After the tubes had stood for 5 minutes, 2 drops of an aged 5 per cent aqueous sodium nitroprusside solution or 1 drop of a 0.2 per cent sodium nitrite solution was added. The characteristic blue color developed at once. After 2 minutes, readings were made in an Evelyn colorimeter with the 6 cc. aperture and Filter 540. The results were plotted, concentration against readings. The curve approximates a straight line up to 0.04 mg.; then the slope changes abruptly, becoming asymptotic at 0.1 mg. concentration. The readings for the curve with nitroprusside differed slightly from those of the curve with sodium nitrite, but they gave the same results when the solutions were referred to their respective curves.

Determination of Tryptophane in Casein—Tryptophane determinations were made on casein digests prepared by two methods, alkaline hydrolysis and enzymatic digestion.

The alkaline hydrolysis was effected by boiling the casein for 6 hours with 5 N sodium hydroxide. Tryptophane was determined on aliquots of the hydrolysate by following each of the three different modifications referred to above. Determinations made at room temperature, with either hydrogen peroxide or sodium nitrite, gave values corresponding to 1.10 to 1.28 per cent of tryptophane, respectively. Determinations made at 85° and the readings compared with those of the standard curve obtained at 85° gave a value of 1.26, but when compared with the curve obtained at room temperature, a value of 0.68 per cent was obtained. Determinations made on a casein digest prepared by the rapid method of Sullivan, Milone, and Everitt, in which the tryptophane was not free, gave a value of 1.1 per cent when compared with the standard curve for room temperature; but when compared with the standard curve for 85° the value corresponded to 2.2 per cent.

TABLE I

Maximum Color Readings for Pure Tryptophane Developed at Room Temperature and at 85° with H_2O_2 and $NaNO_2$

Tryptophane	H_2O_2, room temperature	$NaNO_2$, room temperature	H_2O_2, 85°
mg			
0.0	100	100	100
0.02	91.5	91.5	96
0.04	84.0	84.5	92
0.06	80.0	81.0	87
0.08	79.0	79.0	83

A partially hydrolyzed solution of casein was prepared by digestion with papain according to the procedure referred to later. Tryptophane determinations made on this digest at room temperature and at 85° gave values of 1.11 per cent and 1.01 per cent, respectively, when referred to the room temperature curve, but 2.11 per cent when determined at 85° and referred to the 85° curve. Determinations made on a more extensively hydrolyzed casein, prepared by Dr. Wooley by digestion with pepsin, trypsin, and erepsin and determined at 85°, gave values of 0.58 and 1.28 per cent when referred to the room temperature curve and the 85° curve, respectively, but when determined at room temperature, gave a value of 1.10 (Table II).

The results of the foregoing experiments show that, when the long method of May and Rose or the short method of Sullivan, Milone, and Everitt is used, two considerations must be taken into account. When the tryptophane is free in the protein hydrolysate, it can be determined at elevated temperature and the results compared with the color developed with pure tryptophane at elevated temperature, by using hydrogen peroxide or on long standing without hydrogen peroxide. However, when the tryptophane is bound in peptide union, the color produced cannot be compared with the color obtained at elevated temperature. For consistent results it is best first to prepare the hydrolysate without the color reagent present, and then to develop the color at room temperature and compare the reading with that of the standard curve for room temperature.

TABLE II

Tryptophane Determinations on Completely and Partially Hydrolyzed Casein with Color Developed at Room Temperature and at 85°

Method of hydrolysis	Color developed at room temperature	Color developed at 85°	
		Referred to 85° standard curve	Referred to room temperature standard curve
	per cent	*per cent*	*per cent*
5 N NaOH	1.20	1.26	0.68
HCl (short method of Sullivan, Milone, and Everitt)		2.20	1.10
Digestion with papain	1.11	2.11	1.01
Digestion with pepsin, trypsin, and erepsin	1.10	1.28	0.58

The above considerations are in agreement with the findings of Shaw and McFarlane (9) and they give the additional information that accurate results can be obtained by developing the color at room temperature with the May and Rose reagent, provided that a suitable oxidizing reagent is used. Hydrogen peroxide cannot be used because the color developed fades. However, the color developed with sodium nitrite, or with a 5 per cent aqueous solution of sodium nitroprusside, remains permanent for 1 hour. The sodium nitroprusside solution should be prepared at least a week before it is used. That a freshly prepared solution is not effective suggests that a trace of nitrogen oxide, due to decomposition of the nitroprusside, may be the active agent.

Determination of Tryptophane in Various Proteins and Food Materials—Having established that the maximum color for free tryptophane could be developed at room temperature comparable to its equivalent of tryptophane in the protein, the procedures described were applied to the determination of this amino acid in a number of available isolated and purified proteins. It was of interest to find out whether the procedures, with whatever modifications might prove necessary, could be successfully applied also to food materials such as meals and whole seeds.

The presence of carbohydrate material in most naturally occurring protein foods precludes the use of alkali hydrolysis, because of the formation of dark and colored solutions which obviously render satisfactory colorimetric determinations impossible. Furthermore, complicating chemical reactions take place between the amino acids and carbohydrates or their derivatives. A simple and rapid method was developed for preparing solutions of the nitrogenous matter in meals and flours containing a minimum of carbohydrate, and which can be used directly for colorimetric tryptophane determinations. The method is applicable also for preparing solutions of isolated proteins. By digesting the material at pH 8 with an enzyme solution, 97 to 98 per cent of the nitrogenous material goes into solution, leaving the starch and fiber in suspension.

The enzyme solution was prepared as follows: 2 gm. of commercial papain were shaken for 2 minutes with 100 cc. of water, and the mixture filtered. 10 cc. of the clear filtrate were used for each determination. The tryptophane content of the enzyme solution, 1.1 per cent, was found to be the same whether determined on the above preparation or after autodigestion overnight with 10 drops of sodium cyanide solution. Blanks for the tryptophane were run with each series of protein determinations.

In preparing the protein solutions, 1 to 3 gm. portions of the material, depending on its nitrogen content, were introduced into an Erlenmeyer flask. There were then added 35 cc. of 0.05 N sodium hydroxide, 10 cc. of the papain solution, and 10 drops of a 5 per cent sodium cyanide solution. The flasks were stoppered and allowed to remain in an oven at 70° overnight. A small amount of Celite was then added. After the flasks were shaken, the suspension was filtered and the Celite was washed on the filter with water. The clear light yellow solution was then diluted with water to a volume of 100 cc.

The tryptophane content of a number of isolated proteins and of foodstuffs as determined by the chemical procedure is given in Table III. There are also included for comparison values for the same materials determined by microbiological methods. In this collaborative work, aliquots of the same hydrolysates and papain digests prepared and analyzed by the chemical method in our laboratory were also analyzed by Dr. Wooley and

Dr. Sebrell by the microbiological methods.[1] Similarly, aliquots of enzymatic digests and hydrolysates, prepared and analyzed in Dr. Wooley's laboratory, were analyzed in our laboratory by the chemical method. The results obtained for both isolated proteins and foodstuffs by the two methods are in close agreement, with the exception of those obtained with the sodium hydroxide hydrolysates. Tryptophane cannot be satisfactorily determined by the microbiological method on alkali hydrolysates of protein because of partial racemization of the amino acid by the alkali during hydrolysis. The organism can utilize only the *l*-tryptophane.

TABLE III

*Tryptophane Content of Some Isolated Proteins and Foodstuffs Determined by Chemical and Microbiological Methods**

The percentages are calculated on a moisture-free basis.

Material	N	Determined chemically on papain hydrolysates	Analyses made on pepsin, trypsin, and erepsin digests†		Analyses made on NaOH hydrolysates	
			Determined chemically	Determined microbiologically	Determined chemically	Determined microbiologically
Arachin	18.30	0.90	0 97	1.08	0.70	0.38
Conarachin	18 20				1.02	0.46
Casein (Labco)	14.42	1.20	1.20	1.20	1.10	0.47
Cottonseed globulin.	18.00	1.29			1.24	0.60
Georgia velvet bean globulin	16.70	0.60	0.73	0.73	0.69	0.34
Glycinin (soy bean)	17.50				1.01	0.44
Lactalbumin	15.40	1.72	1.79	1.79	1.74	0.75
Lima bean globulin	15.10	1.01	0.89	0.90	1.01	0.60
Ox muscle	16.00	1.30	1.18	1.17	1.24	0.45
Phaseolin (navy bean)	16.10	0.50	0.38	0.38	0.70	0.25
Squash seed globulin	18.50				1.88	0.76
Wheat bran albumin	15 40				2.31	1.30
Corn germ (defatted)	3 74	0.23	0.26	0.24	0.33	0.22
Cottonseed flour (Proflo)	9.88	0.70	0.64	0.63	0.89	0.36
Peas (black-eyed)	4.21	0.23	0.22	0.24		
Peanut flour.	9.85	0.56	0.56	0.55	0.59	0.28
Soy bean flour	8.81	0.62	0.62	0.55	0 87	0.36
Wheat (whole)	2.14	0.16	0.14	0.13		
“ germ (defatted) .	6.58	0.30	0.33	0.36	0.40	0.33

* The microbiological determinations were made by Dr. Wooley.

† The pepsin, trypsin, and erepsin digests were prepared by Dr. Wooley.

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[1] Owing to the fact that the papain digests were only partially hydrolyzed, the results obtained by the microbiological methods were low and are not included in Table III for comparison.

It is of interest to note that, if the higher percentages of tryptophane recorded in the literature for casein and other proteins are divided by two, the results in most cases will agree fairly closely with those given in Table III.

SUMMARY

A rapid colorimetric method for the determination of tryptophane in isolated proteins and in foods is described. The tryptophane values found are in close agreement with those obtained microbiologically for the same materials by Wooley and Sebrell.

BIBLIOGRAPHY

1. May, C. E. and Rose, E. R., *J. Biol Chem* , **54,** 213 (1922).
2. Holm, G. E., and Greenbank, G R , *J Am. Chem Soc* , **45,** 1788 (1923).
3. Jones, D. B., Gersdorff, C. E. F , and Moeller, O., *J Biol. Chem* , **62,** 183 (1924–25).
4. Sullivan, M. X , Milone, H S , and Everitt, E L., *J. Biol Chem* , **125,** 471 (1938).
5. Milone, H. S., and Everitt, E. L , *Proc Soc Exp. Biol. and Med* , **51,** 82 (1942).
6. Shaw, J. L D., and McFarlane, W. D , *Canad. J. Res., Sect B* , **16,** 361 (1938).
7. Block, R. J , and Bolling, D , The determination of the amino acids, Minneapolis, revised edition (1940).
8. Greene, R. D., and Black, A , *Proc Soc. Exp Biol. and Med* , **54,** 322 (1943).
9. Shaw, J. L D., and McFarlane, W D., *J. Biol. Chem.,* **132,** 387 (1940).

THE COLORIMETRIC DETERMINATION OF FRUCTOSAN IN PLANT MATERIAL

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The wide distribution of fructosan in the Compositae, Gramineae, and certain other families and the physiological importance of this polysaccharide in the carbohydrate economy of these plants emphasize the need for a simple, rapid method adaptable to routine determinations. Embodying the convenience of colorimetry, the procedure to be described offers certain advantages over methods now in current use for plant materials.

The usual techniques for the determination of fructosan in plant extracts consist of a preliminary hydrolysis of the polysaccharide, followed by an estimation of fructose on the basis of its reducing power either selectively in the presence of other reducing sugars (1), or after oxidation of aldoses by iodine in alkaline solution (2). Since these methods involve laborious titrimetric or gravimetric manipulations, their application on a large scale is highly restricted. This consideration in conjunction with the frequently doubtful specificity of the above procedures suggested an investigation of the applicability of a colorimetric determination.

Colorimetric methods for the determination of inulin (and fructose) in blood and urine are based on the color reactions given by the polysaccharide with reagents such as diphenylamine (3–5), skatole (6–8), and resorcinol (9–11). In these procedures the reaction is carried out in strongly acid solution, and the color results from the interaction of the reagent with fructose formed by hydrolysis of the inulin. Sucrose and preformed fructose would thus be included in the determination and, especially in the case of plant material, must be eliminated. Since in most carbohydrate studies on plants it is desirable to determine the mono- and disaccharides as well as the polysaccharides, a separation of the latter class of compounds from the former may be effected by an ethanol extraction.

The procedure as described below for the development and measurement of a colored compound formed by the interaction of fructose and resorcinol is a modification of the method of Roe (9), which is based on the familiar Seliwanoff test for ketoses. A considerable increase in the sensitivity and reproducibility of the method has been effected by a change in the conditions for the color reaction, and through the application of photoelectric colorimetry at a selected region of the spectrum. These improvements permit of greater dilutions of the unknown solution, with virtual elimination of errors due to pigments and non-fructosan chromogens. The adaptation of this method to certain plant materials has been studied in detail, but wide variations in the chemical composition of plant extracts derived from other species argue against its indiscriminate application without due regard to possible sources of error. Procedures for assessing the magnitude of these errors are given.

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Reagents—

Alcoholic resorcinol (Roe). 1 gm. of resorcinol in 1 liter of 95 per cent alcohol.

30 per cent HCl (Roe). 1 volume of distilled water in 5 volumes of concentrated HCl.[1]

Fructose standard. Stock solution, dissolve 100 mg. of fructose in 100 ml. of saturated aqueous benzoic acid solution. This solution is kept in the refrigerator and is used to prepare the working standard by diluting 4 ml. to 100 ml.

Procedure

Preparation of Samples—Plant tissues are preserved by placing them in boiling alcohol or by drying in a convection oven at 65–75° with or without a short pretreatment at 90–100° to destroy enzymes. After the dry material is ground, 1.00 gm. samples are continuously extracted with 80 per cent ethanol for 6 hours, and dried in the thimble. The alcoholic extract may be analyzed for ethanol-soluble sugars in the usual manner.

Extraction of Fructosan—The dry extracted material is quantitatively transferred to a 100 ml. volumetric flask and about 20 ml. of water added. After being held in a water bath at 95–100° for 10 minutes, the tissue will be "wet" and can be washed into the bottom of the flask with about 70 ml. of water. The flask is then replaced in the bath for another 30 minutes with occasional swirling to dissolve the fructosan completely, and is finally removed and cooled to room temperature.

Clarification—If the solution is colloidal in character or pigmented, saturated neutral lead acetate is added until no further precipitate forms. The contents are then made to 100 ml., mixed, and filtered into a flask containing dry potassium oxalate. The resulting lead oxalate is removed by a second filtration. If the original solution is clear and only faintly colored, no treatment other than filtration may be necessary, especially if the solution is to be greatly diluted.

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[1] Variations in the strength of this reagent, due to differences in the HCl content of "concentrated hydrochloric acid," may be eliminated by adjusting the solution to a definite specific gravity; for example, to 1.1600. This will tend to minimize differences in photometric readings arising from slight variations in the strength of the above reagent.

Color Development—5 ml. of the extract (diluted to contain 1 to 8 mg. of fructose per 100 ml.), 5 ml. of alcoholic resorcinol, and 15 ml. of 30 per cent HCl are pipetted into a test-tube. Similarly 5 ml. of water, and 5 ml. of a standard fructose solution prepared from the stock and containing 4 mg. of fructose per 100 ml., are placed in separate tubes, and the resorcinol and acid added. After the contents are mixed, the tubes are placed in an 80° water bath for 20 minutes, cooled to room temperature, and the color density determined in a photoelectric colorimeter with a green (540 $m\mu$) filter. The reagent blank is used to establish the reference setting. In the Klett-Summerson colorimeter (research model) equipped with a solution cell affording a 2.5 mm. light path through the test liquid, Beer's law holds up to a concentration of about 40 mg. per cent of fructose. It is recommended, however, that tubes supplied with the instrument be used in routine work because of the added convenience and increased sensitivity thereby attained. Under these conditions Beer's law appears to obtain up to about 8 mg. per cent of fructose concentration, but individual instruments vary somewhat in this respect. From the reading of the standard is obtained a factor by which readings of the unknowns are converted to concentrations of fructose. Inulin is not used as a primary standard, because the chromogenic value varies with the source and the method of preparation.

EXPERIMENTAL

Preparation of Sample—Identical samples of a fructosan-containing tissue (guayule stems) were prepared by plunging them in boiling alcohol, and by drying for 12 hours at 65° and at 85° in a convection oven with and without a 30 minute pretreatment at 100°. There was no significant difference in the reducing sugar-sucrose ratio or the fructosan content of any of the samples thus prepared. However, the method used in practice must be based upon the nature of the material under consideration, and no general procedure can be recommended for all plants.

Alcohol Extraction—With both ground plant material and inulin as test substances, alcohol at concentrations ranging from 70 to 95 per cent by volume has been studied as extracting medium. At the 80 per cent concentration recommended reducing sugars and sucrose are completely removed in a 6 hour period, but less than 1 per cent of the fructosan present is extracted. Typical curves indicating the distribution of fructose (all forms) in alcohol and water extracts of guayule roots at various alcohol extraction times are shown in Fig. 1. Extraction end-points are attained in 4 to 5 hours.

Clarification—The possible loss of fructosans on treatment of aqueous extracts with neutral lead acetate has been studied in a recovery experiment. To a hot water extract of a ground sample of guayule tissue (previously extracted with 80 per cent ethanol) were added varying amounts of inulin. The solutions were then clarified as described in the procedure, and fructosan determinations carried out on the resulting clear filtrates. Recoveries are shown in Table I, with all values within the ±5 per cent range of reproducibility of the method. The volumes of the ground tissue and lead precipitates are negligible for purposes of this determination.

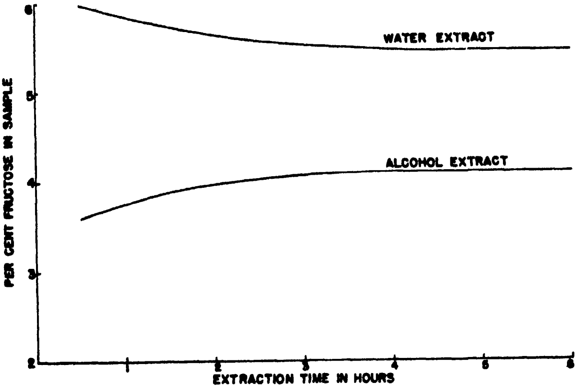

FIG. 1. Curves showing the distribution of fructose (all forms) in alcohol and subsequent hot water extracts of guayule tissue at various alcohol extraction times. The data are expressed as per cent fructose in the dry sample.

TABLE I

Effect of Clarification with Neutral Lead Acetate on Recovery of Inulin Added to Plant Extracts (Guayule) Containing 3.5 Mg of Fructosan per 100 Ml

Inulin added	Inulin recovered	Recovery of added inulin
mg	*mg*	*per cent*
7.70	7.60	99
3.79	3.92	103
1.94	1.89	98
0.77	0.73	95

The use of charcoal as a clarifying agent is attended by certain restrictions. As Roe has shown, fructose is not adsorbed by charcoal from solutions acid to pH 3.1. Fructosan (inulin), however, is lost upon charcoal treatment of its solutions even in strong acid. It is feasible to hydrolyze the fructosan in water extracts by making the solution 0.15 N in HCl and heating in a water bath at 80° for 15 minutes. Charcoal clarification with high grade acid-washed decolorizing carbon may then be carried out without significant error. If this procedure is used, the standard should be carried through a similar treatment.

Color Development—With a photoelectric colorimeter, it was observed that the 8 minute heating time recommended by Roe failed to give stable color densities after cooling. A study of the relation of color density to heating time indicated that the reaction was essentially complete after 20 minutes at 80° (Fig. 2). This heating period yields color densities that change after cooling at the negligible rate of about 1 per cent per hour. Shorter heating times show considerably less stability.

Interference—The relatively short water extraction time (40 minutes) and the subsequent lead acetate clarification preclude the presence of high concentrations of the more insoluble polysaccharides in the filtrate taken for analysis. However, the presence of starch, water-soluble pentosans, and similar highly organized carbohydrates which would yield glucose, galactose, or pentoses under the conditions for color development cannot always be ignored. The degree of interference introduced by such substances has been studied by adding known amounts of the sugars to standard fructose solutions and measuring the resulting errors. The data thus obtained indicate that additive errors of less than 3 per cent are incurred by the presence of galactose up to 3 times, glucose up to 4 times, and pentose up to 10 times the concentration of fructose present.

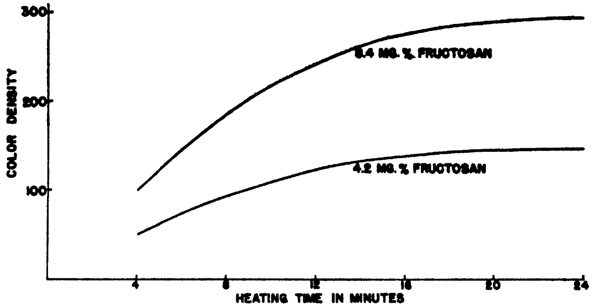

FIG 2. The relationship between color density and heating time for the reaction of fructosan with resorcinol reagent.

An estimate of the interference due to polysaccharides other than those yielding fermentable sugars on hydrolysis and to non-carbohydrate chromogens may be made as follows: A suitable aliquot of the water extract is made 1.0 N in H_2SO_4 and heated in a boiling water bath for 2 hours. The solution is then neutralized, made to volume, and filtered. To a portion of this hydrolysate is added 0.2 volume of a yeast suspension made by washing bakers' yeast at the centrifuge until the supernatant is perfectly clear and suspending the packed cells in an equal volume of phosphate buffer, pH 6.8. After an incubation period of 3 hours at 37°, the yeast is centrifuged down and the clear supernatant analyzed for its chromogen content. In most cases this blank value will be found negligible, or sufficiently constant to obviate routine determinations on each sample.

Comparison of Resorcinol Reaction with Hypoiodite Method—In Table II are shown data comparing fructosan values obtained by the resorcinol method with reducing sugar values following hydrolysis and iodine oxidation of aldoses in alkaline solution (2). The somewhat lower results obtained with the resorcinol method might be ascribed to the greater specificity of this procedure.

TABLE II

Comparison of Results by Colorimetric Method with Reducing Sugar Values after Hydrolysis and Iodine Oxidation of Aldoses

Sample No	Fructosan content (rye-grass)*	
	Colorimetric method	Reducing value after hydrolysis and oxidation of aldoses
1	1.5	1.7
2	7.6	9.1
3	9.9	10.8
4	12.5	13.1

* Rye-grass samples and the data of the last column were kindly supplied by Dr. J. T. Sullivan, United States Department of Agriculture. The values are expressed in per cent based on the dry weight.

DISCUSSION

The foregoing method was developed primarily for a study of carbohydrate metabolism in guayule, *Parthenium argentatum*. All parts of this plant have been successfully analyzed by the procedure outlined, and no difficulty was experienced in applying the technique to several other species containing fructosan. However, because of the widely varying chemical constitution of different plant groups, it is recommended that when the method is used for the first time on a given species the clarified water extract be analyzed for starch by the amylase method or by starch-iodide colorimetry (12). If the glucose equivalent of the starch present is sufficiently high (4 times the approximate fructosan concentration) to introduce a significant error, appropriate corrections must be made, or the starch digested with amylase and the resulting products fermented out with yeast. If representative blank values determined as indicated in the discussion of interference are high and variable, routine estimations of their magnitude are indicated.

It is to be noted that the hot water extraction may be preceded by an extraction at room temperature with water or dilute alcohol for the determination of dextrins and "levulins." Starch may be determined in the presence of fructosan by the method of Nielson (12).

SUMMARY

1. A procedure is outlined for the colorimetric determination of fructosan in plant material. The quantitative reaction is based on the formation of a colored compound between resorcinol and fructosan under conditions modified from those described by Roe.

2. The method may be expected to yield values reproducible to within ±5 per cent.

3. Sources of error peculiar to plant tissues are discussed, and procedures for determining their magnitude are given.

BIBLIOGRAPHY

1. Official and tentative methods of analysis of the Association of Official Agricultural Chemists, Washington, 5th edition (1940).
2. Norman, A. G., Wilsie, C. P., and Gaessler, W G., *Iowa State Coll. J. Sc.*, **15**, 301 (1941).
3. Corcoran, A. C., and Page, I. H., *J. Biol Chem.*, **127**, 601 (1939).
4. Alving, A. S., Rubin, J., and Miller, B. F., *J. Biol. Chem.*, **127**, 609 (1939).
5. Harrison, H. E., *Proc. Soc. Exp. Biol. and Med.*, **49**, 111 (1942).
6. Jordan, R. C., and Pryde, J., *Biochem. J.*, **32**, 279 (1938).
7. Reinecke, R. M., *J. Biol. Chem.*, **142**, 487 (1942).
8. Ranney, H., and McCune, D. J., *J. Biol. Chem.*, **150**, 311 (1943).
9. Roe, J. H., *J. Biol. Chem.*, **107**, 15 (1934).
10. Steinitz, K., *J. Biol. Chem.*, **126**, 589 (1938).
11. Hubbard, R. S., and Loomis, T. A., *J. Biol. Chem.*, **145**, 641 (1942).
12. Nielson, J. P., *Ind. and Eng. Chem., Anal. Ed.*, **15**, 176 (1943).

A SPECIFIC ENZYMATIC METHOD FOR THE DETERMINATION OF CREATINE AND CREATININE IN BLOOD*

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Specific enzymatic methods for the determination of creatinine in blood serum and plasma (1) and of creatine-creatinine in tissues (2) have been reported. The specificity of these methods is dependent on the use of adaptive enzymes present in the NC soil bacteria which have been grown on media containing creatine or creatinine as the sole source of carbon and nitrogen (3). The adaptive powers of these bacteria are indeed striking, since they can also produce adaptive enzymes which specifically destroy nicotinic acid when cultured on media which contain nicotinic acid as the chief source of carbon and nitrogen. This ability to produce nicotinic acid-decomposing enzymes has been utilized in the development of a specific method for the determination of "true nicotinic acid" in blood (4).

The specific enzymatic estimation of creatinine in blood has revealed that almost all of the chromogen in normal blood plasma, serum, and spinal fluid that gives the Jaffe reaction is true creatinine (1), and that endogenous creatinine is excreted almost entirely by filtration alone under normal physiological conditions (5). As a result of the specific estimation of creatine in tissues it has been demonstrated that about 90 per cent of the apparent creatine in skeletal muscle, testis, and brain is true creatine, but that only a small fraction of the apparent creatine in intestinal muscle, kidney, and liver is true creatine (6). The need for a specific method for the determination of creatine in blood has been evident since the reports of the early workers (7–9) that there were only traces of creatine in plasma. Recently, Peters (10) who has modified the colorimetric estimation of creatine-creatinine in blood plasma and urine has reemphasized the desirability of a specific enzymatic method for the analysis of blood creatine.

Method

The procedures used for the determination of creatinine are similar to those already described in previous publications (1, 2). The light transmission of serum filtrates treated with alkaline picrate under rigidly controlled conditions is measured in a photoelectric colorimeter before and after exposure of samples to the action of adaptive bacterial enzymes which specifically destroy creatinine.

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The estimation of creatine is based on the quantitative conversion of creatine in serum filtrates to creatinine under exactly specified conditions of pH, time, and temperature, and the subsequent determination of the material thus formed, that gives the Jaffe reaction, before and after exposure of the samples to enzymatic action as in the determination of creatinine.

Apparent Creatinine—1:5 tungstic acid filtrates of plasma or serum are prepared with 5 per cent sodium tungstate and 0.34 N H_2SO_4. Serum is preferred, since it consistently gives slightly higher values than plasma. The pH of the protein-free filtrates is adjusted to pH 7.0 with 0.1 N HCl, brom-thymol blue being used as external indicator. To duplicate aliquots of 8 cc. of the clear filtrate in Evelyn S tubes are added 4 cc. of freshly prepared alkaline picrate (prepared at 25°). The colors are allowed to develop at 25° for 12 minutes. The light transmission of the solutions is determined in the Evelyn photoelectric colorimeter, the galvanometer being set at 100 with the blank solution. The creatinine equivalents are then determined by reference to calibration lines obtained on pure solutions of creatine. The filter which gives the maximum absorption for dilute solutions of creatinine is composed of Wratten Filter 75 plus Rubicon Filter 9785. The value thus obtained may be designated as T_2, as suggested in an earlier publication (2).

True Creatinine—To duplicate 8 cc. aliquots of filtrate in 125 cc. unstoppered Erlenmeyer flasks are added 0.5 cc. of M phosphate buffer, pH 7.0, a suitable volume of freshly assayed bacterial suspension,[1] and water to 10 cc. Reagent blanks containing the bacterial suspension are similarly prepared. These are incubated at 37° for 45 minutes, and then centrifuged at high speed to remove the bacterial cells; 8 cc. aliquots are then transferred to Evelyn S tubes and carried through the procedure described above. The value thus obtained is designated as R_2, or residual chromogen. The difference $T_2 - R_2$ is true creatinine.

Total Apparent Creatine and Creatinine—Creatine is determined as creatinine after dehydration with hot acid to creatinine. To 8 cc. aliquots of clear filtrate in glass-stoppered test-tubes is added 1.0 cc. of 2.5 N HCl, and the tubes autoclaved at 120–125° at 20 pounds pressure for 1 hour. These conditions of time, pH, and temperature were found to be adequate for quantitatively converting aqueous solutions of creatine to creatinine.

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[1] The activity of the bacterial enzymes has been greatly increased by limiting the period of incubation of the cultures to 48 hours instead of 72 hours (until one-half of the creatine or creatinine in the medium is destroyed).

It is necessary to be careful in building up and releasing the pressure so as not to dislodge the stoppers. After the tubes are removed from the autoclave and cooled, 10 cc. of 2.5 N NaOH (free of carbonate) are added to each tube. One set of duplicate aliquots is then carried through the colorimetric procedure as outlined above for creatinine, except that 5 cc. of alkaline picrate are added to 10 cc. of autoclaved filtrate. The values obtained here are designated as T_1. Another set of duplicate 8 cc. aliquots receives 0.5 cc. of M phosphate buffer and a suitable volume of bacterial suspension and is treated as in the procedure for true creatinine. The creatine equivalents in both instances are derived by reference to calibrations obtained on unheated creatinine solutions to which has been added the same amount of acid, base, and buffer used in the analysis for creatine.

TABLE I

Concentration of True Creatine and Creatinine in Normal Human Serum

All values are given in micrograms per cc.

	T_2 Apparent creatinine	R_2 Residual chromogen	True creatinine	T_1 Total chromogen after autoclaving	R_1 Total residual chromogen	Apparent creatine as creatinine	True creatine as creatinine
Serum	11.7	1.7	10.0	17.3	3.8	5.6	3.5
	12.8	2.6	10.2	19.0	4.9	6.2	3.9
	11.5	1.8	9.7	15.5	3.0	4.0	2.8
	10.4	2.3	8.1	16.1	3.7	5.7	4.3
	10.3	2.1	8.2	15.2	3.9	4.9	3.1
	7.6	1.4	6.2	14.1	2.6	6.5	5.3
	10.7	1.3	9.4	19.0	3.4	8.3	6.2
Plasma	11.3	2.1	9.2	15.3	4.1	4.0	2.0
Cells	16 0	9.5	6.5	51.6	12.5	35.6	32.6
Plasma	9.8	2.2	7.6	15.7	4.5	5.9	3.6
Cells	15.3	10.1	5.2	48.2	14.2	32.9	28.8

The value obtained in the analysis after treatment with the bacterial suspensions is designated as R_1. The difference of $T_1 - R_1$ gives the concentration of total true creatine and creatinine. True creatine as creatinine is then found by taking the difference of $(T_1 - R_1) - (T_2 - R_2)$.

Results

The results of the specific technique on the sera and corpuscles of normal individuals are presented in Table I. The plasma and serum of normal humans contain from 4.0 to 8.3 γ per cc. of apparent creatine as creatinine. Approximately 70 per cent of the apparent creatine is true creatine. The erythrocytes in the two cases studied contained considerably greater amounts of true or apparent creatine than did plasma. The apparent serum creatine value is considerably higher than the values reported by Wu and agrees fairly well with the data of Tierney and Peters (11).

The same technique for creatine estimation may be applied to urine. Certain precautions such as the omission of preservatives must be followed.

SUMMARY

A specific enzymatic method for the determination of true creatine and creatinine in human serum is described. The specificity of the technique is dependent on the use of enzymes present in washed bacterial cells which have been cultured on media containing creatine or creatinine as the source of carbon and nitrogen.

The true serum creatine level is found to be about 70 per cent of the apparent creatine content. Erythrocytes contain much greater amounts of true creatine than does plasma.

The author is indebted to the Valentine Meat Juice Company, Richmond, Virginia, for the creatine and creatinine used in this study.

BIBLIOGRAPHY

1. Miller, B. F., and Dubos, R., *J. Biol. Chem.*, **121**, 447, 457 (1937).
2. Miller, B. F., Allinson, M. J. C., and Baker, Z , *J Biol. Chem* , **130**, 383 (1939).
3. Dubos, R., and Miller, B. F., *J. Biol. Chem* , **121**, 429 (1937).
4. Allinson, M. J. C., *J. Biol. Chem.*, **147**, 785 (1943).
5. Miller, B. F., and Winkler, A. W., *J. Clin. Invest.*, **17**, 31 (1938).
6. Baker, Z., and Miller, B. F , *J. Biol. Chem.*, **130**, 393 (1939).
7. Wu, H., *J. Biol Chem* , **51**, 21 (1922).
8. Wilson, D. W., and Plass, E. D., *J. Biol. Chem.*, **29**, 413 (1917).
9. Hunter, A., and Campbell, W. R., *J. Biol. Chem.*, **32**, 195 (1917).
10. Peters, J. H., *J. Biol Chem* , **146**, 179 (1942).
11. Tierney, N. A., and Peters, J. P., *J. Clin. Invest.*, **22**, 595 (1943).

THE BIURET REACTION OF PROTEINS IN THE PRESENCE OF ETHYLENE GLYCOL

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The biuret method offers some advantages as a simple, rapid photometric method for the determination of proteins. In the methods which have been proposed, precipitation of cupric hydroxide gives rise to certain difficulties (1, 2). It occurred to us that the precipitates might be avoided by the addition of a reagent which would form a compound with copper which was soluble in alkali, but of such a nature that the copper could still react with protein. Ethylene glycol has been examined and found suitable, and with the use of this substance it is possible to prepare a single, alkaline copper solution which may be added to the protein for the development of the biuret color.

Absorption Spectra—The absorption spectrum of the complex of cupric ion and ethylene glycol is shown in Curve B of Fig. 1. The concentration of ethylene glycol was 8.0 ml. per 100 ml., and that of sodium hydroxide was 2.5 gm. per 100 ml. (hereafter concentrations expressed in these terms will be referred to as per cent). The values of $E_{1\,\text{cm}}^{1\%}$ are calculated in terms[1] of the total copper concentration, and were obtained with solutions in which the concentration ranged from 0.001 to 0.12 per cent. One maximum is observed at 630 mμ ($E_{1\,\text{cm}}^{1\%} = 4.7_5$) and another at 297 m$\mu$ ($E_{1\,\text{cm.}}^{1\%} = 64._5$), with a minimum in the region from 380 to 430 mμ.

The absorption spectrum of the egg albumin-copper complex is shown in Curve A of Fig. 1, and was determined by subtracting the blank due to the copper and ethylene glycol reagent and that due to the protein in the ultraviolet region. The values of $E_{1\,\text{cm}}^{1\%}$ are calculated in terms of the protein concentration, and maxima are found at 545 mμ ($E_{1\,\text{cm}}^{1\%} = 2.5_8$) and at 300 m$\mu$ ($E_{1\,\text{cm}}^{1\%} = 11._5$), with a minimum at 430 mμ. The position of the maximum reported by Sizer (3) is 552 mμ. The position of the maximum found for mixed serum proteins in the present study is also essentially the same as for egg albumin, whereas the absorption maximum reported by Robinson and Hogden (2) is 560 mμ.

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[1] The notation is that commonly used, according to which $\log I_0/I = E_{1\,\text{cm.}}^{1\%}\, cd$, where I_0/I represents the original intensity divided by the intensity of the transmitted light, c is the concentration in gm. per 100 ml., and d is the depth of the solution in cm.

The displacement of the maximum by protein makes it possible to correct the values obtained in the visible region for the absorption due to copper not combined with protein. The absorption in alkaline mixtures containing copper, ethylene glycol, and protein at 750 mμ may be taken as due to the copper-ethylene glycol complex. $E^{1\%}_{1\,\text{cm.}}$ is 2.0 at this wave-length. At 545 mμ, the value of $E^{1\%}_{1\,\text{cm.}}$ for copper-ethylene glycol is 3.1_6. The value for the absorption due to the protein-copper complex at 545 mμ would be obtained by subtracting 1.57 times the optical density at 750 mμ from the optical density at 545 mμ. Similar corrections may be made at other wave-lengths.

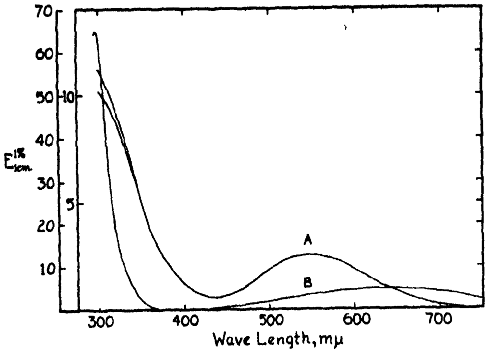

FIG. 1. Curve A, the absorption spectrum of the egg albumin-Cu complex. Values for the scale of extinction are on the right, and are calculated in terms of the protein concentration. The values above 430 mμ were obtained with 0 134 and 0.235 per cent protein. The upper branch of the curve below 430 mμ was obtained with 0 0134 per cent and the lower branch with 0 0335 per cent protein. Curve B, the absorption spectrum of copper sulfate and ethylene glycol in alkaline solution. The extinction is calculated in terms of the copper concentration, and is referred to the scale on the left.

When such a correction is made for the absorption due to the copper which is not combined with protein, the relationship between optical density and protein concentration is still not strictly linear. In Table I, the results obtained with egg albumin, and absorption at 530 and 750 mμ, are given (a wave-length of 545 mμ would have been somewhat more suitable than 530 mμ). The values of $E^{1\%}_{1\,\text{cm.}}$ are seen to decrease somewhat as the concentration increases. This may actually be a deviation from Beer's law, or may depend, at least in part, upon an inadequate correction when the blank amounts to as much as 50 per cent of the total reading. The curve for the absorption of the protein-copper complex given in Fig.

1 would therefore be somewhat different for different concentrations. The values used were the averages for concentrations of 0.134 and 0.235 per cent protein in the region from 350 to 750 mμ. The difference between the values of $E_{1\,\text{cm.}}^{1\%}$ is in the neighborhood of 3 per cent at these two protein concentrations.

In the spectral region below 350 mμ, the correction for uncombined copper cannot be so readily made, since both the copper and protein concentrations are diminished to bring the readings into a suitable range. The values which have been plotted were obtained by subtracting the optical densities of alkaline copper-ethylene glycol and of alkaline protein solutions of the concentrations used in the biuret reaction. The upper branch of Curve A was obtained with 0.0134 per cent egg albumin and the lower branch with 0.0335 per cent egg albumin.

TABLE I

Relation between Egg Albumin Concentration and Extinction Coefficient or Colorimeter Readings

Protein	Optical density at 1 cm			$E_{1\,\text{cm}}^{1\%}$, 530 mμ	Colorimeter reading, Filter 54	Increase in colorimeter reading for 1 per cent protein
	530 mμ	750 mμ	Due to biuret color, 530 mμ			
gm. per 100 ml.						
0					69	
0.034	0.210	0.083	0.093	2.7_7	116	1380
0.067	0.287	0.079	0.175	2.6_1	161	1370
0.101	0.373	0.078	0.263	2.6_1	205	1350
0.134	0.445	0.074	0.340	2.5_4	251	1360
0.168	0.522	0.073	0.419	2.5_0	292	1330
0.201	0.598	0.068	0.502	2.5_0	335	1320
0.235	0.671	0.065	0.579	2.4_7	377	1310
0.269	0.735	0.061	0.649	2.4_1	408	1260
0.302	0.796	0.058	0.714	2.3_7	452	1270

Effect of Copper Concentration—The concentration of copper needed to produce maximum color development is dependent upon the concentration of protein. In Fig. 2 the optical density is shown as a function of the copper concentration for three concentrations of human plasma protein. It will be seen that the absorption is independent of protein concentration when the copper concentration is low. This indicates that all of the copper is bound to protein, and permits us to calculate the value of $E_{1\,\text{cm.}}^{1\%}$ referred to the concentration of copper in the protein-copper complex. This value is $18._8$ at 545 mμ. The concentration of copper needed to give an excess for plasma protein concentrations up to about 0.15 per cent is approximately 0.05 per cent.

Stability of Protein-Copper Complex—As in the studies previously reported (2), we have found the color to be stable, but that it requires some time to develop fully. When egg albumin is used, about 90 minutes must be allowed for development of the color. After this time, the color is constant for at least 20 hours. The situation is complicated in the case of plasma, since a turbidity develops in about 1 hour. As this turbidity also develops in plasma treated with the same concentration of alkali, there does not seem to be any way of entirely eliminating the changes with time in the case of plasma, and a comparison with the results obtained on egg albumin is correspondingly inexact.

Use of Modified Biuret Method in Determination of Protein Concentrations—The relation between protein concentration and optical density has already been noted in the case of egg albumin. In those experiments, and in the ones to be presented, the final concentration of copper was 0.043 per cent, the ethylene glycol concentration was 8 volumes per 100, and the sodium hydroxide concentration was 4.3 per cent. Table I also gives the results obtained with solutions of egg albumin with the Klett photoelectric colorimeter. The readings were all made at 2 hours. Table II gives the values for human plasma and trichloroacetic acid precipitates of plasma, for which the readings were made at 30 to 40 minutes. During the period between 25 and 45 minutes, the changes in readings are relatively small, and reasonably consistent values can be obtained at definite times.

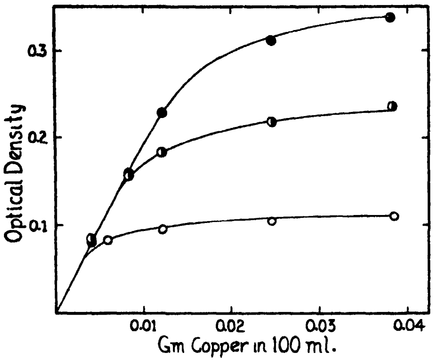

FIG. 2. The effect of copper concentration upon the optical density in solutions containing different concentrations of plasma protein. The protein concentrations are 0.149 ●, 0.099 ◑, and 0.050 ○ per cent The optical density is that due to the protein-Cu complex at 545 mμ.

It will be seen that the optical density is not strictly proportional to the protein concentration. $E^{1\%}_{1\,\text{cm.}}$ decreases somewhat with increasing protein concentration, although the correction for the decreased copper available for combination with ethylene glycol has been made. This would again indicate some deviation from Beer's law. It will also be noted that the values for plasma protein are about 15 per cent lower than those for egg albumin at equal concentrations, although this may be in part due to incomplete development of color in the case of plasma. The albumin and globulin fractions of plasma have not been compared in this investigation, but it does not seem likely that they will differ more in this method than in previous methods (2). At any rate, no obvious difficulties are encountered when this procedure is applied to the filtrate from the globulin precipitation by the Howe method, and sodium sulfate does not alter the color intensity. Even ammonium ion has relatively little effect, and may be neglected when the ammonia nitrogen does not exceed the protein nitrogen.

TABLE II

Relation between Plasma Protein Concentration and Extinction Coefficient or Colorimeter Readings

Protein	$E^{1\%}_{1\,\text{cm}}$, at 530 mμ Protein-Cu complex		Increase in colorimeter reading for 1 per cent protein	
	Plasma	Pptd plasma	Plasma	Pptd plasma
mg. per 100 ml.				
0.050		2.15		1280
0.050	2.28		1220	1240
0.099		2.11		1240
0.100	2.22		1210	1180
0.149		2.07		1230
0.150	2.24		1240	1170
0.197		2.08		1200
0.201	2.21		1190	1140
0.251			1180	1110

Inspection of the absorption curves in Fig. 1 shows that the sensitivity of the method might be increased by using the near ultraviolet. For this purpose, we have chosen to use a wave-length somewhat longer than that of the region of maximum absorption, since the correction due to residual copper will be reduced more than the absorption due to the protein-copper complex. Corrections due to other absorbing materials which might be present in solutions other than those of purified proteins will also generally be smaller. The results of some measurements made on egg albumin at 320 mμ are given in Table III. The copper concentration was decreased to 0.0047 per cent and the ethylene glycol to 4 volumes per 100 for these measurements. The sensitivity of the method is increased between 3 and 4 times over that in the visible region. The same increase in sensitivity may be achieved in the case of plasma, although this advantage will be somewhat offset by the greater influence of turbidity at these wave-lengths.

EXPERIMENTAL

Measurements of Optical Density—The instrument used was a Beckman quartz photoelectric spectrophotometer. The instrument had been recently rechecked by the manufacturer, and readings have been made on potassium chromate at regular intervals. The wave-length settings have always checked to within 1 mμ, and the density readings to within 1 per cent. The tungsten lamp was used as a light source throughout, and the band width varied from about 2 mμ in the visible to about 4 mμ at the shortest wave-lengths.

TABLE III

Relation between Egg Albumin Concentration and Optical Density in Near Ultraviolet

Protein	Optical density, 1 cm, 320 mμ			Protein-Cu $E_{1\,\text{cm}}^{1\%}$, 320 mμ
	Total	Protein in alkali	Protein-Cu	
mg. per 100 ml.				
0	0.091			
6.7	0.162	0.005	0.066	10.1
13.4	0.225	0.011	0.123	9.2
20.1	0.286	0.016	0.179	8.9
26.8	0.348	0.022	0.235	8.8
33.5	0.413	0.027	0.295	8.8

The photoelectric colorimeter was a Klett-Summerson, and was used with the manufacturer's Filter 54, having a transmission centered at 540 mμ.

Protein Solutions—The egg albumin was prepared from frozen egg white, by precipitation with ammonium sulfate. The initial precipitate of egg albumin was not crystalline, but the material was obtained crystalline in the subsequent three precipitations. The ammonium sulfate was removed by dialysis against running water for 48 hours and against 20 volumes of distilled water for 24 hours. The residual ammonia nitrogen was 0.25 per cent. The concentration of protein was determined by drying to constant weight at 105°.

The human plasma was a commercial product, supplied through the courtesy of the Hyland Laboratories. The protein concentration was obtained from the dry weight of the heat-coagulated material after two washings with 50 per cent alcohol, one with 95 per cent alcohol, and one with ether. These values were checked by obtaining the nitrogen precipitated by 7.5 per cent trichloroacetic acid and multiplying by 6.25.

Combined Copper Reagent—In some of the earlier experiments, it was noted that solutions containing sodium hydroxide, copper sulfate, and ethylene glycol developed a marked turbidity and eventually deposited a red precipitate. This process was accelerated by light or heat, and was also observed in samples containing plasma. It seems likely that reducing impurities were present in the ethylene glycol, and that the high concentrations of glucose in the commercial plasma were responsible for additional reduction of the copper. The effect of reduction in the blanks can be eliminated by mixing the combined reagent and heating it to complete the reaction. A satisfactory reagent for work in the visible region may be prepared by mixing 100 ml. of ethylene glycol, 40 ml. of 60 per cent NaOH, and 50 ml. of 4 per cent $CuSO_4 \cdot 5H_2O$. This solution is diluted to about 400 ml. and heated until precipitation is complete. After the cuprous hydroxide is filtered off, sufficient sodium hydroxide is added to make the final concentration 10 to 11 per cent. This reagent appears to be stable for at least several months. We have used 10 ml. in a total volume of 25 ml. for protein determinations.

It is obvious that reagents of a different composition may prove more useful in special cases. For the work in the ultraviolet region, a dilute copper reagent was made in a similar way, except that the final ethylene glycol concentration was decreased to 1 volume in 10, and only enough copper was added to give a very faint blue color after the mixture had been heated. The final concentration of sodium hydroxide was also lowered to 6.3 per cent. This reagent contained about 0.012 per cent copper, and was used in the amount of 10 ml. in a final volume of 25 ml.

In both of the reagents suggested, there is obviously a considerable excess of ethylene glycol, and additional copper can be added without precipitation. The concentration of alkali may also be varied over a rather wide range, and we have observed no differences in the absorption of the copper-protein complex with sodium hydroxide concentrations between 2 and 6 per cent in the final solutions. However, the higher concentrations of alkali increase the rate of development of turbidity in the plasma protein solutions. We feel that a final concentration of alkali between 2 and 4 per cent is most satisfactory. It is probably the safest procedure to place solutions containing plasma in the dark while the biuret color is allowed to develop. The reducing effect of glucose in plasma may thus be avoided.

DISCUSSION

The introduction of an additional component into the biuret reaction is obviously objectionable on the grounds that interactions of an unknown nature may be expected, and the interpretation of the results may be further complicated. The finding that the value of $E_{1\,\mathrm{cm.}}^{1\%}$ is somewhat dependent upon concentration, even at protein concentrations at which the copper is still in excess, is not in agreement with the observation of Robinson and Hogden (2) on filtered solutions which do not contain ethylene glycol. The influence of an inadequate correction for the absorption of copper not combined with protein may have to be considered as a factor in the present study. The difference between positions reported for the absorption maximum can be more readily interpreted in terms of the uncombined copper present in the solutions obtained with the usual procedure. Since the absorption of this copper is farther toward the red end of the spectrum, but overlaps the absorption of the protein-copper complex, the combined absorption curve will be broadened and shifted toward the longer wave-lengths.

In our own experience, we have found the use of ethylene glycol justified, and we believe that the possibility of avoiding precipitation of cupric hydroxide or elimination of the filtration outweighs the disadvantages. It may also be possible to apply this method to a more detailed study of the reaction between cupric ion and proteins and related compounds. The dissociation constant of the complex and the relation between the absorption in the near ultraviolet and visible regions would be of some interest.

The purchase of the spectrophotometer was made possible by a grant from the Rockefeller Foundation.

SUMMARY

1. The biuret reaction is modified by the introduction of ethylene glycol, which prevents the precipitation of cupric hydroxide. A single reagent may be employed, which gives optically clear solutions when mixed with protein.

2. Deviations from Beer's law, which may be as large as 10 per cent for a 4-fold change in protein concentration, have been observed under these conditions. Such deviations can readily be corrected for, and do not seriously impair the usefulness of the method.

3. The sensitivity of the biuret method may be further increased by making use of the greater absorption of the protein-copper complex in the region around 320 mμ.

BIBLIOGRAPHY

1. Kingsley, G. R., *J. Biol. Chem.*, **131**, 197
2. Robinson, H. W., and Hogden, C. G., *J. Biol. Chem.*, **135**, 707 (1940).
3. Sizer, I. W., *Proc. Soc. Exp Biol. and Med.*, **37**, 107 (1937).

THE METABOLISM OF THE METHYLATED PURINES

I. THE ENZYMATIC DETERMINATION OF URINARY URIC ACID*

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Myers and coworkers (1) have suggested that caffeine, theophylline, and theobromine may be oxidized in the 8 position by the animal body to give rise to methylated uric acids. Certain of these methylated derivatives react, as does uric acid with phosphotungstic acid, in an alkaline medium to form blue-colored reduction products. Since some of the methyluric acids are precipitated along with uric acid by both ammoniacal silver and acid silver lactate, the presence of such methylated derivatives in urine would give rise to high values for uric acid by both the direct and silver precipitation procedures. Consequently it is impossible by these methods of analysis to decide whether the increased reduction of the phosphotungstic acid reagent by a urine collected after the ingestion of caffeine or theophylline is due to extra uric acid formed by the demethylation and oxidation of these compounds or to the presence of methyluric acids.

The present paper describes an enzymatic procedure which will distinguish between uric acid and its methylated derivatives. The proposed method is similar in principle to that of Blauch and Koch (2) and of Bulger and Johns (3) for blood uric acid in that the enzyme uricase is used for the destruction of uric acid. Keilin and Hartree (4) have reported that uricase is a highly specific enzyme and does not oxidize any of the mono-, di-, or trimethyluric acids.

A suitable aliquot of the urine is allowed to react with arsenophosphotungstic acid in an alkaline medium. The blue color which is obtained is measured in the Evelyn photoelectric colorimeter and is referred to as the total color, since the reduction is due in part to the presence of compounds other than uric acid. A second aliquot of urine is buffered at the optimum pH for uricase activity and incubated with the enzyme for 2 hours at the optimum temperature. All of the uric acid is destroyed, but the non-uric acid reducing substances including the methyluric acids are not oxidized by the uricase. The solution is then treated with sodium tungstate and sulfuric acid to remove protein and the residual color is determined in the filtrate as in the direct procedure. The difference between the total color and the residual color is taken as a measure of the uric acid destroyed; *i.e.*, true uric acid.

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* A preliminary report was presented before the Michigan Academy of Science on March 26, 1943.

† The Rackham Arthritis Research Unit is supported by the Horace H. Rackham School of Graduate Studies of the University of Michigan.

Reagents—

Urea-cyanide (5).

Arsenophosphotungstic acid (6).

10 per cent sodium tungstate.

0.66 N sulfuric acid.

Borate buffer (pH 9.2).

Approximately 0.1 N NaOH.

0.04 per cent thymol blue indicator.

Standard uric acid. A uric acid standard is prepared as described by Folin (7), except that the indicator is omitted. Suitable dilutions of this standard are used to obtain the data for the calibration curve described below. Since the formaldehyde and sulfuric acid present in the regular Folin standard are not favorable for uricase activity, a solution of uric acid in lithium carbonate is used for all procedures which involve the standardization or testing of uricase activity. This special lithium carbonate standard contains 1.000 gm. of uric acid and 0.6 gm. of lithium carbonate per liter of distilled water and is prepared according to Folin's directions (7) with the omission of the formaldehyde, indicator, and sulfuric acid. This special standard and the dilute Folin standard are very unstable and should be prepared fresh every day.

Uricase powder. 5 pounds of fresh beef kidneys from which all superficial fat has been removed are ground in a meat chopper. The resulting tissue is placed in a large, wide mouthed bottle and washed with tap water to remove the blood and urine, as suggested by Truszkowski and Gubermanowna (8). Small portions of the resulting material are homogenized with an approximately equal weight of benzene in a Waring blendor, and the combined portions returned to a similar type of bottle. After the addition of 2 volumes of cold acetone, the protein material settles to the bottom of the container, and is filtered through cheese-cloth or a laboratory towel and squeezed dry. The solid material containing the crude uricase is again suspended in about 3 times its weight of acetone, is allowed to settle, and is then filtered. This step is repeated until the resulting powder is thoroughly dehydrated and defatted. The material is then spread on towels and allowed to dry in air overnight. The resulting powder is screened through a 40 mesh sieve and stored in a vacuum desiccator.

250 mg. of the uricase powder should give no blank color when carried through the incubation procedure and subsequent color reaction described below. The activity of the preparation should be tested by its power to

oxidize the special lithium carbonate uric acid standard. 3 to 4 mg. of this uric acid should be completely destroyed after incubation with 250 mg. of the enzyme powder.

Calibration Curve—Aliquots of the dilute Folin standard ranging from 10 to 120 γ are transferred to 50 cc. volumetric flasks, and the volumes adjusted to 25 cc. with distilled water. After the addition of 2.5 cc. of urea-cyanide and 1 cc. of arsenophosphotungstic acid, the flasks are made to volume immediately and thoroughly mixed. The color is allowed to develop for exactly 30 minutes, and is then read in the Evelyn colorimeter with Filter 690.

The center setting of the instrument is determined in the usual manner with a blank tube containing the reagents alone. The combination of the arsenophosphotungstic acid of Benedict (6) and the urea-cyanide of Christman and Ravwitch (5) was chosen because a negligible blank color is obtained which does not increase during the time required to read a series of unknown solutions. Combinations of the reagents employed in the other commonly used uric acid methods give a gradually increasing color due to the reagents themselves and therefore are not desirable for use on the Evelyn colorimeter.

Under the conditions described, the color follows the law of Beer and Lambert over a range from 40 to 120 γ and a K factor may be determined and used for the conversion of optical density to concentration of uric acid. Below 40 γ such a relationship does not hold and the concentration of unknowns must be read directly from the calibration curve.

Determination of Total Color—The 24 hour urine sample is diluted to 2 liters with water. A 5 cc. aliquot is transferred to a 250 cc. volumetric flask and diluted to volume with distilled water. 5 to 10 cc. of this solution are placed in a 50 cc. volumetric flask, the volume is adjusted to 25 cc. with distilled water, and the color developed and read as described under the calibration curve.

Determination of Residual Color—2 to 5 cc. of the original 2 liters of urine are transferred to a 50 cc. Erlenmeyer flask, to which are added 10 cc. of water and a few drops of thymol blue. The amount of alkali required to produce a definite blue tint is determined. A similar aliquot of urine without the indicator is then transferred to a 50 cc. volumetric flask and the predetermined amount of alkali added. 250 mg. of uricase powder and 5 cc. of borate buffer are added and 10 cc. of water used to wash down the sides of the flask. The mixture is incubated in a water bath at 45° for 2 hours. After the addition of 1 cc. of 10 per cent sodium tungstate and 1.5 cc. of ⅔ N sulfuric acid, the flask is made to volume with distilled water, and the contents are thoroughly mixed and filtered. 10 cc. of the filtrate are transferred to a 50 cc. volumetric flask, the volume is adjusted to 25 cc.

with water, and the color is developed and read as described under the calibration curve.

When samples of urine smaller than the total 24 hour output are to be determined, aliquots proportional to those described above are used for both the direct and incubation procedures.

Results

A number of substances known to occur in human urine such as adenine, guanine, xanthine, the methylxanthines, allantoin, various phenols, and amino acids were studied to determine whether they would interfere with the uric acid estimation. None of these materials gives a color with the reagents nor does their presence prevent the complete oxidation of uric acid by uricase. The color produced by the reducing action of uric acid is neither enhanced nor diminished by the presence of these materials in urine in any reasonable amounts.

TABLE I

Non-Uric Acid Materials Which React with Arsenophosphotungstic Acid to Give Blue-Colored Reduction Products

Compound	Chromogenic value as compared to uric acid
	per cent
1-Methyluric acid	105.1
1,3-Dimethyluric acid	50.9
Ascorbic acid	44.0
Resorcinol	32.3
3-Methyluric acid	31.9
Ergothioneine	23.8
1,7-Dimethyluric acid	2.2
Cystine	0.54
Glutathione	0.51
3,7-Dimethyluric acid	0.35
1,3,7-Trimethyluric acid	Trace
7-Methyluric acid	"

A group of compounds which do give a color with the reagents is listed in order of their decreasing chromogenic value in Table I. However, the color due to these substances does not interfere with the determination of uric acid, since it appears in both the direct and residual readings. Blauch and Koch (2) gave values showing that ascorbic acid, resorcinol, glutathione, and cystine are not broken down by the enzyme preparation and that the color due to their presence is the same before and after incubation. Keilin and Hartree (4) showed that uricase is specific for uric acid and does not act upon the methylated uric acids. The present study indicates that the color produced by ergothioneine and the methyluric acids is not diminished by the incubation with uricase. Although Keilin and Hartree reported that the methyluric acids act as competitive inhibitors with uric acid for the enzyme, under the conditions specified complete oxidation of uric acid occurs in the presence of methyluric acids added in amounts equivalent to as much as 10 gm. per 24 hour urine sample.

98 to 101 per cent of uric acid added to urine was recovered, as determined by the direct procedure. A similar order of recovery was found upon the addition of various amounts of uric acid to the filtrate of the urine after incubation with uricase. A comparison of the uricase procedure with two standard silver precipitation methods, the ammoniacal silver precipitation of Benedict and Hitchcock (9) as modified by Christman and Ravwitch (5) and the acid silver lactate precipitation of Folin (10), is given in Table II. In order to show that extra uric acid added to urine was completely destroyed, the equivalent of 150 and 300 mg. of uric acid per 24 hours was added to urine Sample 4 to give Samples 5 and 6. The close agreement of the residual colors of Samples 4, 5, and 6 shows that the enzyme completely oxidized the added uric acid.

TABLE II

Comparison of Uricase Method with Silver Precipitation Methods of Folin and of Benedict and Hitchcock

All values are expressed in terms of uric acid per 24 hours.

Urine sample No	Uricase method			Silver isolation	
	Color before uricase action	Residual color after uricase action	True uric acid	Ammoniacal silver	Acid silver lactate
	mg	*mg*	*mg*	*mg*	*mg*
1	642.8	85.8	557.0	563.0	561.4
2	369.5	77.3	292.2	298.6	296.4
3	618.5	62.2	556.3	548.1	
4	672.4	85.9	586.5	591.0	
5	822.3*	85.5	736.8		
6	972.5†	86.3	886.2		

* The equivalent of 150 mg per 24 hours of extra uric acid was added to Sample 4.

† The equivalent of 300 mg. per 24 hours of extra uric acid was added to Sample 4.

As shown in Table II the proposed enzymatic method agrees very well with the two silver precipitation procedures and offers no advantages over them when used on urines collected from subjects on a diet low in caffeine or theophylline (free of coffee or tea). However, it does possess the distinct advantage over previously reported methods in its ability to distinguish between uric acid and the methylated uric acids, and thus offers itself as a new tool for the investigation of the metabolism of the methylated xanthines. As will be reported in a subsequent paper, the ingestion of caffeine or theophylline gives rise to chromogenic materials in the urine which are not destroyed by uricase, but which are precipitated, at least partially, by both ammoniacal silver and acid silver lactate. Consequently such urines will give high values for uric acid by both the direct colorimetric procedures and the indirect colorimetric procedures involving a preliminary precipitation with silver salts.

After the work discussed here and in Paper II following was completed, Schaffer (11) published a procedure similar to the one just described. However, the color reagents, the preparation of the enzyme powder, and the conditions of incubation differ considerably from the present method. Schaffer reports that according to his procedure an average of 3.5 per cent of the color value determined by Folin's direct method is due to non-uric acid materials. The present study indicates that subjects on a diet low in the methylated xanthines excrete a rather constant amount of non-uric acid, chromogenic material. This material varies from 60 to 90 mg. per 24 hours when expressed in terms of uric acid, and amounts to 10 to 20 per cent of the total color, depending upon the concentration of the latter. Apparently the possibility of larger amounts of non-fermentable chromogenic substances in the urine of tea and coffee drinkers which give rise to much larger residual color values was not considered by the above author. A study of the non-uric acid reducing materials excreted after the ingestion of theophylline and caffeine is reported in Paper II (12).

SUMMARY

A procedure has been developed for the determination of urinary uric acid in the presence of methylated uric acids, which is based on the specificity of the enzyme uricase for uric acid.

The method described gives values which agree closely with those obtained by silver precipitation procedures on urines from subjects on diets low in methylated xanthines. In such urines, only 80 to 90 per cent of the color developed by the direct procedures is due to uric acid.

The advantage of such a procedure in the study of the metabolism of the methylated xanthines is pointed out.

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BIBLIOGRAPHY

1. Myers, V. C., and Wardell, E. L., *J. Biol. Chem.*, **77**, 697 (1928). Myers, V. C., and Hanzal, R. F., *Am. J. Physiol.*, **90**, 458 (1929).
2. Blauch, M. B., and Koch, F. C., *J. Biol. Chem.*, **130**, 443 (1939).
3. Bulger, H. A., and Johns, H. E., *J. Biol. Chem.*, **140**, 427 (1941).
4. Keilin, D., and Hartree, E. F., *Proc. Roy. Soc. London, Series B*, **119**, 114 (1936).
5. Christman, A. A., and Ravwitch, S., *J. Biol. Chem.*, **95**, 115 (1932).
6. Benedict, S. R., *J. Biol. Chem* , **51**, 187 (1922). Benedict, S. R., and Franke, E., *J. Biol. Chem.*, **52**, 387 (1922).
7. Folin, O., *J. Biol. Chem.*, **86**, 179 (1930).
8. Truszkowski, R., and Gubermanowna, S., *Biochem. J.*, **29**, 2787 (1935).
9. Benedict, S. R., and Hitchcock, E. H., *J. Biol. Chem.*, **20**, 619 (1915).
10. Folin, O., *J. Biol. Chem.*, **101**, 111 (1933).
11. Schaffer, N. K., *J. Biol. Chem.*, **153**, 163 (1944).
12. Buchanan, O. H., Christman, A. A., and Block, W. D., *J. Biol. Chem.*, **157**, 189 (1945).

THE METABOLISM OF THE METHYLATED PURINES

II. URIC ACID EXCRETION FOLLOWING THE INGESTION OF CAFFEINE, THEOPHYLLINE, AND THEOBROMINE

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The wide-spread use of coffee, tea, and cocoa as beverages and their marked stimulatory and diuretic action have led to numerous investigations concerning the fate of caffeine (1,3,7-trimethylxanthine), theophylline (1,3-dimethylxanthine), and theobromine (3,7-dimethylxanthine) in the animal body. Although it is generally accepted that these compounds are readily absorbed after oral or parenteral administration, only small amounts of the drugs are excreted unchanged (1–3) The isolation of the monomethylxanthines from the urine of both humans and experimental animals (2, 4–6) after the administration of the above compounds definitely showed that the di- and trimethylxanthines are at least partially demethylated. Kruger and his associate (7) suggested that in man the methyl group in position 3 is the most labile, while that in position 7 is the most stable, and that the mono- and dimethylxanthines normally found in human urine can be accounted for by the removal of the methyl group occupying position 3 from the methylated xanthines of the food.

With the partial demethylation of the di- and trimethylxanthines thus demonstrated, the question arose as to the possibility of a conversion of a portion of these compounds to uric acid by means of complete demethylation and an oxidation in the 8 position. Much of the earlier work concerning such a conversion of the methylated xanthines to uric acid is contradictory and open to criticism because of the use of dogs and rabbits as experimental animals. Both of these species excrete allantoin, and not uric acid, as the chief end-product of purine metabolism. However, most of the early work on human subjects, in which the analyses were made by the inaccurate gravimetric and titrimetric methods for uric acid then available, indicated that there was no increase in the urinary excretion of uric acid after the ingestion of caffeine, theophylline, or theobromine (8–10).

The development of colorimetric procedures for the determination of uric acid by Folin and his associates (11) and Benedict and Hitchcock (12)

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resulted in the reinvestigation of the problem and the formulation of the opposite point of view; *i.e.*, that the methylated xanthines *do* give rise to an increased excretion of uric acid in man. In 1916, Benedict (13), using the new ammoniacal silver precipitation procedure of Benedict and Hitchcock (12), reported an experiment in which the ingestion of 1 gm. of caffeine per day for 4 days led to an increased output of uric acid amounting to 100 to 160 mg. per day above control values. A short time later, Mendel and Wardell (14), using the same colorimetric method, reported that the ingestion of coffee, tea, or caffeine by their subjects was followed by an increased uric acid excretion. This increased excretion appeared to be proportional to the quantity of caffeine ingested and was equivalent to the amount of uric acid which would be obtained by the demethylation and subsequent oxidation of from 10 to 15 per cent of the ingested caffeine. Clark and de Lorimier (15) in 1926 studied both the blood levels of uric acid and the urinary excretion of uric acid after the ingestion of caffeine and theobromine. Both compounds caused an increase in blood uric acid levels as measured by Benedict's direct method (16). Caffeine administration was followed by a rise in uric acid excretion, as determined by the direct method of Benedict and Franke (17), while theobromine appeared to diminish the rate of excretion of uric acid. They concluded that the methylxanthines are not oxidized directly and that the increased uric acid excretion after caffeine ingestion is probably due to a stimulation of general metabolism.

Myers and Wardell (18) found that the ingestion of caffeine and theophylline by human subjects resulted in an increased uric acid excretion, as determined by both the direct colorimetric method of Benedict and Franke (17) and the silver isolation procedure of Benedict and Hitchcock (12). They confirmed the report of Clark and de Lorimier that theobromine causes no such increase. Myers and Wardell made the suggestion that a portion of the methylated xanthines might be oxidized in the 8 position before being completely demethylated, thus giving rise to methyluric acids. It was pointed out that the oxidation of theophylline (1,3-dimethylxanthine) in position 8 would give rise to 1,3-dimethyluric acid, while both oxidation and removal of the more labile methyl group in the 3 position would result in the formation of 1-methyluric acid. Both of these methylated derivatives of uric acid give the color reaction with the uric acid reagents. On the other hand, by a similar mechanism, theobromine (3,7-dimethylxanthine) would give rise to 3,7-dimethyluric acid or to 7-methyluric acid. Neither of these latter compounds causes any appreciable reduction of phosphotungstic acid. The formation of methylated uric acids by such an oxidation and demethylation mechanism was offered as a possible explanation for the differences observed in "uric acid" excretion following the administration of theophylline and theobromine.

Myers and Hanzal (19) later studied the metabolism of the methylated xanthines as well as the methylated uric acids in the Dalmatian dog. The oral administration of either caffeine or theophylline resulted in an increased excretion of uric acid as determined by colorimetric methods. Theobromine ingestion had little effect on the uric acid excretion. After the administration of theophylline, materials having the reducing properties of uric acid were isolated by precipitation with silver salts. After the removal of the silver and recrystallization, crystals resembling those of 1,3-dimethyluric acid were obtained. Similar studies made after the ingestion of theobromine suggested the formation of 3,7-dimethyluric acid.

These authors found that the methyluric acids which have a methyl group in position 7 give only traces of color with the phosphotungstic acid reagents. No increase in uric acid was observed after the ingestion of this group of compounds by the Dalmatian dog. Those methylated uric acids which *do not* have a methyl group attached in the 7 position all reduce phosphotungstic acid. The oral administration of this group of methylated uric acids to the Dalmatian hound caused an increase in uric acid excretion. However, the extra color observed could be ascribed to the elimination of the unchanged methyluric acids. In other words, the extra color was proportional to that which would be anticipated if the methyluric acids were excreted unchanged. From these results the workers concluded that the methylated derivatives of uric acid are "apparently quite stable in the animal organism."

Thus, the work of Myers *et al.* suggests that the increase in uric acid excretion observed after the administration of caffeine and theophylline is probably not due to uric acid itself but rather to its methylated derivatives. Certain of the methyluric acids do resemble uric acid in many of their properties and would be included as uric acid by both the direct and silver precipitation procedures. Consequently it is impossible by either of these methods of analysis to decide whether the extra uric acid is actually uric acid or whether it is due to the formation and excretion of the methylated uric acids. In order to investigate this problem further, an enzymatic procedure (20) has been developed for the specific determination of uric acid in the presence of its methylated derivatives. It is the purpose of this paper to report the application of this method to a study of the metabolism of the three methylated xanthines commonly found in the human diet.

EXPERIMENTAL

Metabolic studies were carried out on five different individuals to investigate the effect of the administration of caffeine, theophylline, and theobromine on uric acid excretion. In the first series of experiments, the general plan was to place the subject on a purine-free diet for a period of 9 days. This was divided into a 3 day control period, a 3 day experimental period during which 1 gm. per day of the methylxanthine in question was fed in two divided doses, and a final 3 day control period. Urine samples were collected over 24 hour periods and analyzed for total phosphotungstic acid-reducing materials, residual reducing materials, and true uric acid by the uricase procedure of Buchanan, Block, and Christman (20). Analyses were also made for uric acid by an adaptation of the ammoniacal silver precipitation method of Benedict and Hitchcock (12) to the Evelyn photoelectric colorimeter with the alkaline urea-cyanide reagent of Christman and Ravwitch (21) and the arsenophosphotungstic acid of Benedict (16, 17). Creatinine determinations were made on all 24 hour samples of urine to check on the completeness of the urine collection. Since these values showed only slight variations from day to day for a single individual, they have not been included in Tables I and II.

Table I contains a summary of the uric acid values for subject O. H. B. after the administration of caffeine, theophylline, and theobromine. This individual does not drink coffee or tea and this fact may explain the differences observed in the results for this subject as compared to those found for the two subjects (Table II) who are habitual drinkers of coffee.

The ingestion of caffeine caused an 84 per cent increase in the total reducing materials, from a 3 day average control value of 435 mg. per 24 hours to an average of 799 for the experimental days. The true uric acid increased only 5 per cent, from an average of 390 to 411 mg. per 24 hours. The striking feature of the experiment was the tremendous increase in residual color; *i.e.*, the phosphotungstic acid-reducing materials which are not oxidized by incubation with uricase. This residual material, measured in terms of uric acid, increased from an average value of 45 mg. per 24 hours for the 3 day control period to an average of 388 mg. per 24 hours for the experimental period, a 760 per cent increase. This rise in residual color is still evident on the day following the last administration of caffeine, probably because the last dose of drug was taken late in the preceding afternoon. It is because of this lag in the excretion of the residual material that the values obtained for the 3 days preceding the ingestion of the methylxanthines have been used in all cases to compute the average control values. In order to prove that sufficient uricase was being employed to destroy all of the uric acid in these experimental urines, known amounts of uric acid were added to these samples and shown to be completely oxidized by the enzyme.

Uric acid as determined by the silver precipitation procedure rose to a level averaging 95 per cent above that found for the control period. If the average for true uric acid for the experimental days (411 mg.) is subtracted from that for the silver precipitation (689 mg.) and the result divided by the average increase in residual color (343 mg.), it is found that about 82 per cent of the extra non-uric acid reducing material is carried down in the silver precipitation fraction. The possibility that this residual color is due to the presence of methylated uric acids and the implications of such a hypothesis will be discussed below.

TABLE I

Effect of Ingestion of Methylated Xanthines on Uric Acid Excretion

Subject, O. H. B ; age, 28, weight, 130 pounds. All values are expressed as mg. excreted in 24 hours.

Date	Uric acid by uricase method			Uric acid by silver pptn	Diet
	Total color*	Residual color*	True uric acid		
1943					
Mar. 29	412	42	370		Low purine
“ 30	442	49	393	341	“ “
“ 31	450	43	407	375	“ “
Apr. 1	719	302	417	621	1 gm caffeine
“ 2	889	454	435	780	1 “ “
“ 3	789	409	380	666	1 “ “
“ 4	497	114	383	439	Low purine
“ 5	429	49	380	377	“ “
“ 6	370	40	330	290	“ “
June 9	370	34	336	352	“ “
“ 10	454	44	410	419	“ “
“ 11	432	42	390	391	“ “
“ 12	850	454	396	549	1 gm. theophylline
“ 13	897	509	388	590	1 “ “
“ 14	910	543	367	635	1 “ “
“ 15	537	212	325	366	Low purine
“ 16	488	60	427	424	“ “
“ 17	415	61	354	364	“ “
1944					
Mar. 9	406	34	372		“ “
“ 10	392	34	358		“ “
“ 11	358	34	324		“ “
“ 12	388	53	334	347	1 gm. theobromine
“ 13	344	55	289	277	1 “ “
“ 14	401	57	344	351	1 “ “
“ 15	414	49	365		Low purine
“ 16	444	55	389	396	“ “

* Expressed in terms of uric acid.

The ingestion of theophylline gave results similar to those obtained for caffeine. The average increases in total color and residual color were 112 and 1155 per cent respectively, while the slight increase in true uric acid was within the experimental error of the method. The silver precipitation procedure showed an average increase of 52 per cent over the control values. By calculations similar to those described for caffeine, it was found that about 45 per cent of the extra non-uric acid reducing material is precipitated by the ammoniacal silver-magnesium mixture.

No significant changes in either total color or true uric acid were found after the administration of theobromine. Only a comparatively slight increase was observed in the residual color. The values obtained by the silver precipitation method agree very closely with those found for true uric acid by the uricase method. Two additional studies with theobromine on other subjects gave values confirming the results reported in Table I.

The effect of the ingestion of caffeine on the uric acid excretion of two other individuals and the results after theophylline dosage in one are shown in Table II. The results show that a considerable variation is found between individuals, which perhaps may be correlated with previous coffee drinking habits or age. The subjects of the experiments reported in Table II are habitual coffee drinkers, whereas the other subject (O. H. B., Table I) does not drink coffee or tea.

Subject A. A. C. showed a 20 per cent average increase in total color and a 187 per cent increase in residual color after the ingestion of caffeine. The true uric acid values decrease slightly but, again, this is within the experimental error of the method. Average values obtained by the silver precipitation procedure are approximately 13 per cent greater than those of the control period. About 80 per cent of the extra residual material is included in the silver-precipitable fraction. The addition of liver to the diet (high purine period in Table II) caused a prompt increase in true uric acid but had only a slight effect on the residual color.

The ingestion of caffeine by W. D. B. resulted in an increase of 227 per cent residual color, but had little effect on the true uric acid excretion. In this experiment the precipitation with silver salts appears to have carried down approximately 100 per cent of the extra residual material. The administration of theophylline caused a definite increase in residual material of 390 per cent on the 1st experimental day. The subject became ill from the effects of the drug on the 2nd day and all values show a marked decrease. However, the residual color was still considerably greater than on the control days. Calculations, based on data obtained for the 1st experimental day only, show that about 44 per cent of the extra residual material was precipitated by the ammoniacal silver reagent.

A summary of the values recorded in Tables I and II shows that the administration of caffeine and theophylline causes a marked increase in a non-uric acid, phosphotungstic acid-reducing material. The so called direct procedures include all of this reducing material as uric acid, while the procedures involving a preliminary precipitation with silver salts appear to carry down with uric acid from 80 to 100 per cent of the extra reducing material excreted after caffeine ingestion and about 45 per cent of that excreted after theophylline administration. The uricase procedure, however, demonstrates that there is no increase in true uric acid after the administration of either caffeine or theophylline. Theobromine causes no significant change in uric acid excretion as measured by either the direct, the silver precipitation, or the uricase method.

TABLE II

Effect of Ingestion of Caffeine and Theophylline on Uric Acid Excretion

All values are expressed as mg. excreted in 24 hours.

	Date	Uric acid by uricase method			Uric acid by silver pptn.	Diet
		Total color*	Residual color*	True uric acid		
	May, 1943					
Subject, A. A. C.; age, 47; weight, 142 lbs.	11	615	69	545	577	Low purine
	12	469	54	415	421	" "
	13	515	56	459	480	" "
	14	628	159	469	541	1 gm. caffeine
	15	647	185	462	571	0.9 gm. caffeine
	16	564	131	433	480	Low purine
	17	565	71	494	514	" "
	18	550	67	482	485	" "
	19	692	79	613	653	High "
	June, 1943					
Subject, W. D. B.; age, 32; weight, 160 lbs.	2	431	75	356	399	Low "
	3	511	73	438	460	" "
	4	409	70	339	347	" "
	5	547	217	330	471	1 gm. caffeine
	6	665	295	370	584	1 " "
	7	601	206	395	542	1 " "
	8	577	104	473	520	Low purine
	9	734	63	661	677	" "
	10	658	66	592	610	" "
	11	534	62	472	470	" "
	12	584	83	501	497	" "
	13	661	341	320	439	1 gm. theophylline
	14	421†	190†	231†	291†	1 " "
	15	469	100	369	372	Low purine
	16	528	63	465	477	" "
	17	534	77	467	481	" "

* Expressed in terms of uric acid.

† The subject became ill from the effects of the drug.

The results of a study of the rate of excretion of uric acid and the non-uric acid reducing materials after the ingestion of caffeine and theophylline are shown in Fig. 1. Subject O. H. B. was placed on a purine-free diet for a period of 11 days. 500 mg. of caffeine were fed on the 2nd and 7th days and 500 mg. of theophylline on the 4th and 9th days. Analyses similar to those previously described were made on urines collected over 2, 3, and 6 hour periods, as indicated in Fig. 1. All values are calculated in mg. of material excreted per hour and are plotted against time in hours.

An hourly variation was observed in the amount of total phosphotungstic acid-reducing materials excreted during the control periods. The values were usually higher from 8 a.m. to 2 p.m. and decreased during the early morning hours, but, in general, average from 15 to 18 mg. per hour. The ingestion of either caffeine or theophylline caused a definite increase in total color during the first 12 hours after administration, which was followed by a marked decrease during the second 12 hour period. It should be emphasized again that these values for total color are the sum of the two components, true uric acid and non-uric acid residual materials. Similar results would be observed if direct procedures for uric acid, such as the Benedict-Franke or Folin's direct method (22), had been used. Clarke and de Lorimier, using the Benedict-Franke method, obtained increases in uric acid excretion similar to those after the administration of caffeine.

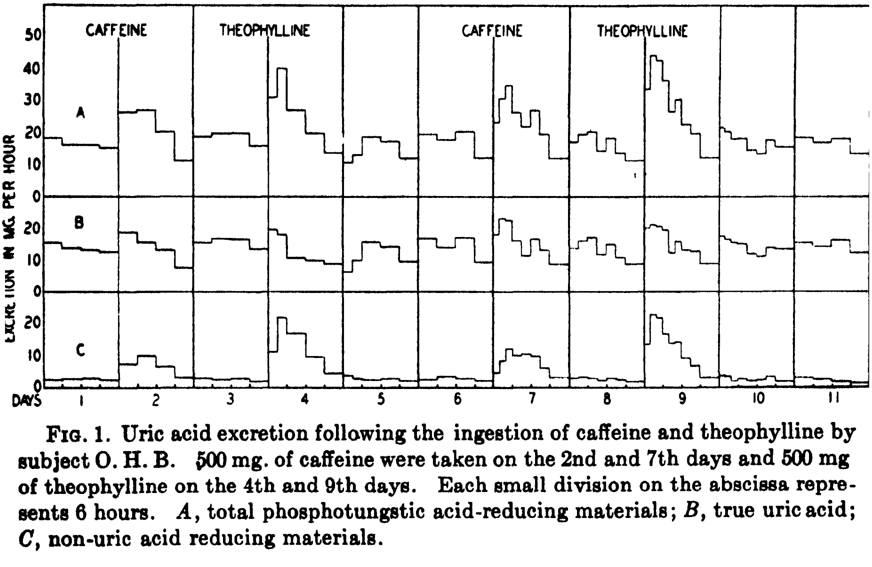

FIG. 1. Uric acid excretion following the ingestion of caffeine and theophylline by subject O. H. B. 500 mg. of caffeine were taken on the 2nd and 7th days and 500 mg of theophylline on the 4th and 9th days. Each small division on the abscissa represents 6 hours. *A*, total phosphotungstic acid-reducing materials; *B*, true uric acid; *C*, non-uric acid reducing materials.

Values for true uric acid ranged from 10 to 18 mg. per hour on the control days. The excretion was usually greater during the portion of the day when the subject was more active and diminished during the early morning hours. The first ingestion of caffeine caused a slight increase in true uric acid during the first 6 hours, but this was followed by a gradual decrease to the very low level of 7.7 mg. per hour during the early morning hours. A similar increase and subsequent decrease were observed after the second administration of caffeine. Theophylline ingestion caused fluctuations in true uric acid resembling those found with caffeine. However, when the total amounts of true uric acid excreted over each 24 hour period are compared, it can be seen that the daily excretion of true uric acid is not significantly changed by the administration of either caffeine or theophylline. The initial increase in true uric acid excretion which occurs during the first 6 to 8 hours after the ingestion of these two methylated xanthines is compensated by a decreased excretion which occurs later in the day. It is suggested that this temporary increase in uric acid excretion is caused by either a stimulation in general metabolism or by the intense diuresis resulting from the ingestion of caffeine and theophylline in such large amounts. All subjects suffered very definite nervous stimulation after dosage with either drug and in the study reported in Fig. 1 the excretion of urine during the 2 hour period immediately following the administration of 500 mg. of theophylline averaged 8.8 cc. per minute as compared to 1 to 2 cc. per minute during the control periods.

The most striking results were those obtained for residual color, as shown in Fig. 1. While the hourly excretion during the control days was relatively constant and averaged from 2 to 4 mg. per hour, marked increases were observed after the ingestion of 500 mg of caffeine or theophylline. The first administration of caffeine resulted in values for residual color of 7.5, 11.1, and 6.8 mg. per hour for each of the three 6 hour periods following dosage. The residual material excreted during the three 2 hour periods immediately following the second ingestion of caffeine amounted to 4.9, 8.4, and 12.2 mg. per hour, respectively. Theophylline produced an even greater rise in non-uric acid reducing materials, as shown by values of 22 mg. per hour for the 3 to 6 hour period following the first dosage, and 23 mg. per hour during the 2 to 4 hour period after the second administration. It can be seen from Fig. 1 that theophylline causes a much greater increase in residual materials and is more prompt in its action than caffeine.

Table III gives the total 24 hour values calculated from the data presented in Fig. 1. The figures listed in the third column show the remarkably consistent values obtained for the non-uric acid reducing materials excreted by an individual on a low purine diet for a number of days, and the very definite increases in this fraction after the ingestion of caffeine and theophylline. The values of 173 and 179 mg. per 24 hours after the ingestion of 500 mg. of caffeine and 283 and 272 mg. after the administration of 500 mg. of theophylline show that the ingestion of a certain amount of methylxanthine causes the excretion of a definite, consistent amount of non-uric acid reducing material by any one individual. From the results given in the fourth column, it can be seen that the excretion of true uric acid is not significantly increased by the administration of either caffeine or theophylline when considered over 24 hour periods.

TABLE III

*Effect of Ingestion of Caffeine and Theophylline on Uric Acid Excretion**

Subject, O.H.B.; age, 29; weight, 130 pounds. All values are expressed as mg. excreted in 24 hours.

Date	Total reducing materials†	Non-uric acid reducing materials†	True uric acid	Diet
Apr., 1944				
19	397	63	334	Low purine
20	509	173	336	500 mg. caffeine
21	442	63	379	Low purine
22	576	283	293	500 mg. theophylline
23	357	67	290	Low purine
24	415	68	347	" "
25	532	179	353	500 mg. caffeine
26	375	66	309	Low purine
27	626	272	354	500 mg. theophylline
28	399	62	337	Low purine
29	395	49	346	" "

* Data from Fig. 1 summarized for 24 hour periods.

† Measured in terms of uric acid.

DISCUSSION

The oral administration of caffeine, theophylline, or theobromine has little effect on the excretion of true uric acid over 24 hour periods, as determined by the uricase procedure. However, the ingestion of caffeine and theophylline does cause the excretion of considerable amounts of a phosphotungstic acid-reducing material which is not oxidized by the enzyme uricase. All of this non-uric acid reducing material is included as uric acid by the direct colorimetric methods for the determination of uric acid, and a portion of the material is carried down with uric acid by the indirect colorimetric procedures involving a preliminary precipitation with silver salts. This non-specificity of the older colorimetric procedures for determining uric acid thus would seem to be the explanation for the numerous reports in the literature of an increased uric acid excretion following the administration of these two methylated xanthines.

Myers and coworkers were the first to suggest the possible formation of methylated uric acids by the oxidation of the methylxanthines. They further showed that certain of the methyluric acids reduce phosphotungstic acid and are precipitated by both ammoniacal silver and acid silver lactate. However, they published no proof of their hypothesis and had no analytical procedure available to distinguish between uric acid and its methylated derivatives. The results obtained by the application of the more specific enzymatic procedure to a study of the problem show that the extra reducing material excreted after the ingestion of caffeine and theophylline is not uric acid, but this method still offers no direct proof as to the identity of the non-uric acid materials.

Ascorbic acid, resorcinol, ergothioneine, glutathione, cystine, and several of the methyluric acids reduce phosphotungstic acid under the alkaline conditions used in the uric acid determinations, and none of these substances is oxidized by incubation with uricase (20). Urine samples collected before and after the administration of caffeine to one of the subjects in the present study were analyzed for ascorbic acid, but no increases were observed. Glutathione and cystine give only 0.51 and 0.54 per cent as much color with phosphotungstic acid as uric acid (20). Therefore nearly 300 gm. of these substances would have to be excreted over a 4 day period to account for the 1500 mg. of extra residual material excreted by O. H. B. after the ingestion of 3 gm. of theophylline. Since large proportions of the residual material are precipitated by ammoniacal silver, resorcinol could not be a source of the residual color. Ergothioneine has a chromogenic value 24 per cent of that of uric acid and is precipitated by ammoniacal silver. Analyses for ergothioneine were not carried out, but the excretion of 6 gm. of this material in 4 days would be required to account for the 1500 mg. of residual material mentioned above.

The methyluric acid hypothesis suggested by Myers and coworkers appears to be the most probable explanation for the increases in residual color. Only three of the methylated uric acids reduce phosphotungstic acid to any appreciable degree (20). Under the conditions of the uricase procedure 1-methyluric acid, 1,3-dimethyluric acid, and 3-methyluric acid give an amount of color equivalent to 105.1, 50.9, and 31.9 per cent respectively of that given by uric acid. Of these three methyluric acids, only the 1-methyluric acid is precipitated by ammoniacal silver under the conditions used throughout this work. It is not precipitated from weak lithium carbonate solutions by ammoniacal silver, but when added to urine is precipitated almost completely along with uric acid.

By the mechanism suggested by Myers *et al.*, caffeine could give rise to all of the tri-, di-, and monomethyluric acids which have methyl groups substituted in the 1, 3, or 7 position. Either the 1,3-dimethyluric acid, 1-methyluric acid, or 3-methyluric acid would have to be formed to account for the reduction of the color reagents. It would appear, then, that at least a portion of the caffeine must be demethylated in the 7 position.

For subjects O. H. B. and A. A. C. about 80 per cent of the extra residual material was precipitated by ammoniacal silver, while nearly 100 per cent was carried down in the experiment on W. D. B. Only 1-methyluric acid is precipitated by this procedure. Therefore, it is suggested that from 80 to 100 per cent of the extra reducing material excreted after caffeine ingestion is 1-methyluric acid, while the remainder is either 1,3-dimethyluric acid, 3-methyluric acid, or a mixture of both compounds. The absolute amount of these compounds which could be formed appears to be subject to individual variations.

1,3-Dimethyluric acid would be formed by the oxidation of theophylline in position 8, while either 1-methyluric acid or 3-methyluric acid would result from a combination of oxidation and partial demethylation. From the results listed in Table I, subject O. H. B. appears to have converted about 50 per cent of the 3 gm. of theophylline ingested into chromogenic methyluric acids. About 45 per cent of the latter was precipitated by ammoniacal silver and therefore would seem to be 1-methyluric acid.

By a simple oxidation in position 8, theobromine would be converted into 3,7-dimethyluric acid. An oxidation and partial demethylation would result in the formation of either 3-methyluric acid or 7-methyluric acid. Of these three possible metabolites only 3-methyluric acid reduces phosphotungstic acid to any appreciable extent. Therefore, the only conclusion which can be made from the present study concerning the metabolism of theobromine is that neither uric acid nor 3-methyluric acid is formed in any significant amount.

The authors appreciate that the final proof that the non-uric acid reducing materials are methyluric acids awaits the isolation of these compounds. After the present metabolic experiments were completed, preliminary work on such isolations was started. Since the entire time of two of the authors was directed to a war project, it was necessary that this work be temporarily postponed.

SUMMARY

The ingestion of caffeine or theophylline by human subjects is followed by a definite increase in the excretion of phosphotungstic acid-reducing materials as measured colorimetrically by both the direct or silver precipitation methods for the determination of uric acid. The administration of theobromine causes no such increase in uric acid excretion.

The fractionation of this chromogenic material into true uric acid and residual reducing materials by the use of the enzyme uricase shows that the true uric acid excretion is increased very little, if at all, over a 24 hour period.

A slight increase in true uric acid does occur during the first 6 hours after the administration of caffeine or theophylline, but a diminished output later in the day compensates for this temporary increase. It is suggested that this slight increase is due to diuresis or a temporary stimulation of metabolism rather than to a conversion of the methylxanthines to uric acid.

The ingestion of caffeine or theophylline causes a very definite increase in the excretion of phosphotungstic acid-reducing materials which are not oxidized by incubation with uricase. Evidence has been presented which suggests that these materials may be 1-methyluric acid, 3-methyluric acid, or 1,3-dimethyluric acid formed by the oxidation and partial demethylation of the methylated xanthines.

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BIBLIOGRAPHY

1. Goodman, L., and Gilman, A., The pharmacological basis of therapeutics, New York, 280 (1941).
2. Albanese, M., *Arch. exp. Path. u. Pharmakol.*, **35**, 449 (1895).
3. Rost, E., *Arch. exp. Path. u. Pharmakol* , **36**, 56 (1895).
4. Bondzynski, S., and Gottlieb, R., *Ber chem. Ges.*, **28**, 1113 (1895); *Arch. exp. Path. u. Pharmakol.*, **36**, 45 (1895); **37**, 385 (1896).
5. Krüger, M., and Schmidt, P., *Ber. chem. Ges.*, **32**, 2677 (1899).
6. Krüger, M., and Salomon, G., *Z. physiol. Chem.*, **24**, 364 (1898); **26**, 350 (1898–99).
7. Krüger, M., *Ber. chem. Ges.*, **32**, 3336 (1899). Krüger, M., and Schmid, J., *Z. physiol. Chem.*, **36**, 1 (1902).
8. Minkowski, O., *Arch. exp. Path. u. Pharmakol.*, **41**, 375 (1898).
9. Burian, R , and Schur, H., *Arch. ges. Physiol.*, **80**, 241 (1900).
10. Krüger, M., and Schmid, J., *Z. physiol. Chem.*, **32**, 104 (1901).
11. Folin, O., and Macallum, A. B., *J. Biol. Chem.*, **11**, 265 (1912). Folin, O., and Macallum, A. B., Jr., *J. Biol. Chem.*, **13**, 363 (1912–13). Folin, O., and Denis, W., *J. Biol Chem.*, **12**, 239 (1912); **13**, 469 (1912–13).
12. Benedict, S. R., and Hitchcock, E. H., *J. Biol. Chem* , **20**, 619 (1915).
13. Benedict, S. R., *J. Lab. and Clin. Med* , **2**, 1 (1916).
14. Mendel, L. B., and Wardell, E. L., *J. Am. Med. Assn.*, **68**, 1805 (1917).
15. Clark, G. W., and de Lorimier, A. A., *Am. J. Physiol.*, **77**, 491 (1926).
16. Benedict, S. R., *J. Biol. Chem.*, **51**, 187 (1922).
17. Benedict, S. R , and Franke, E., *J. Biol Chem.*, **52**, 387 (1922).
18. Wardell, E. L., and Myers, V. C., *Proc. Soc. Exp. Biol. and Med.*, **23**, 828 (1926). Myers, V. C., and Wardell, E. L., *Skand. Arch. Physiol.*, **49**, 189 (1926); *J. Biol. Chem.*, **77**, 697 (1928).
19. Myers, V. C., and Hanzal, R. F., *Am. J. Physiol.*, **90**, 458 (1929). Hanzal, R. F., and Myers, V. C., *Proc. Am. Soc. Biol. Chem.*, *J. Biol. Chem.*, **97**, p. lxix (1932).
20. Buchanan, O. H., Block, W. D., and Christman, A. A., *J. Biol. Chem.*, **157**, 181 (1945).
21. Christman, A. A., and Ravwitch, S., *J. Biol. Chem.*, **95**, 115 (1932).
22. Folin, O., *J. Biol. Chem.*, **101**, 111 (1933).

THE CHEMISTRY OF THE LIPIDS OF TUBERCLE BACILLI

LXXII. FATTY ACIDS OCCURRING IN THE WAX PREPARED FROM TUBERCULIN RESIDUES. CONCERNING MYCOCEROSIC ACID*

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It has been found in previous studies reported from this laboratory (1) that the wax fractions isolated from the human tubercle bacillus, Strain H-37, contain a series of fatty acids combined with a specific polysaccharide (2). In addition to mycolic acid (3), which is the chief acid constituent, small amounts of normal fatty acids occur, such as palmitic, oleic, stearic, and hexacosanoic acids. The presence of tuberculostearic acid and the dextrorotatory phthioic acid has also been observed.

What appears to be of special interest is the occurrence of fatty acids of high molecular weight that are levorotatory. These acids form lead salts that are easily soluble in ether. A levorotatory fatty acid was first encountered in the analysis of the purified wax (1). Later on an acid was isolated which melted at 48–50° and its specific rotation was −6.1° (4). Its molecular weight and composition agreed approximately with the formula $C_{30}H_{60}O_2$. The low melting wax contained in the mother liquors in the purification of the phosphatide (5) contained a levorotatory fatty acid which melted at 37–38°, and the specific rotation of different fractions varied from −7 to −10°. The molecular weight and composition of these acid fractions were in agreement with the formula $C_{31}H_{62}O_2$.

In view of the fact that the purification of the levorotatory fatty acid has been found to be even more difficult than the purification of phthioic acid, the actual composition and specific rotation of the acid cannot be regarded as definitely established. It is moreover possible that homologous or even isomeric forms of the acid may be present. However, judging by observations in this laboratory extending over a period of years, there appears to be no doubt that a levorotatory fatty acid fraction having the ap-

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† Holder of a National Tuberculosis Association Fellowship at Yale University, 1942–44.

The data are taken from the dissertation submitted by Leonard G. Ginger to the Faculty of the Graduate School, Yale University, 1943, in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

proximate composition represented by the formula $C_{30}H_{60}O_2$ is a constant cleavage product of the wax fractions of the human tubercle bacillus, Strain H-37. In order to designate this acid by a characteristic name we propose to call it "mycocerosic acid." Should it be found eventually that more than one such acid occurs, then the prefixes α-, β-, etc., may be applied.

The present investigation concerns itself primarily with a study of the levorotatory mycocerosic acid which was isolated from the wax fraction of cell residues from the preparation of the purified protein derivative PPD (6). As has been indicated in an earlier publication (7), the exact strain of tubercle bacilli used in the preparation of this lot of PPD is unknown, but it was undoubtedly a human strain, although certain differences in fatty acid constituents have been found, as well as differences in the polysaccharide component of the phosphatide.

EXPERIMENTAL

The crude chloroform-soluble wax which served as the starting material in this investigation had been isolated from cell residues, as described in Paper LXVIII of this series (7).

Purification of the Wax—For purification 308.6 gm. of the crude wax were dissolved in 1.5 liters of warm ether and the solution was filtered to remove some filter paper fibers. The solution was cooled in ice water and about an equal volume of methyl alcohol was added slowly with stirring. The precipitate that separated was filtered off and washed with methyl alcohol. The precipitate was redissolved in ether and precipitated by the addition of methyl alcohol. After these operations had been repeated four times, a nearly white powder was obtained which, after it had been dried *in vacuo*, weighed 232.6 gm. The mother liquors and washings were combined and the solvent was evaporated. The residue formed a deep yellowish salve-like mass which weighed 75.5 gm. This fraction was saved for future investigation.

The purified wax softened at 43° and formed a clear melt at 53°, $[\alpha]_D$ in $CHCl_3 = +3.8°$. The substance was nearly saturated, since the iodine number was only 2.3.

Analysis—Found, C 76.49, H 12.85, N 0.10, P 0 12, ash 0.73

The high oxygen value, about 9.3 per cent by difference, suggests the presence of a considerable amount of carbohydrate.

Saponification of the Purified Wax—It has been found in earlier experiments in this laboratory that the tubercle bacillus wax on saponification yields carbohydrate, mycolic acid, certain normal fatty acids whose lead salts are insoluble in ether, levorotatory acids, and small amounts of other acids, such as oleic, tuberculostearic, and phthioic acids, whose lead salts are easily soluble in ether, neutral material, and sometimes glycerol. A method for the separation of these cleavage products was described by Reeves and Anderson (8), and the same procedure was applied in the present case.

For saponification 227.6 gm. of the purified wax were dissolved in about 2 liters of benzene and 500 cc. of normal methyl alcoholic potassium hydroxide were added. After the solution had stood at room temperature for a short time, a precipitate consisting of carbohydrate separated. The solution was warmed, after which the precipitate was filtered off, washed with hot benzene, and purified, as will be described later. To the combined filtrate and washings were added 15 gm. of potassium hydroxide dissolved in 100 cc. of methyl alcohol, and the solution was refluxed in an atmosphere of nitrogen for 72 hours. The reaction mixture was concentrated *in vacuo* to a volume of about 700 cc., diluted with 1.5 liters of water, acidified with hydrochloric acid, and extracted five times with ether.

The aqueous solution was neutralized with potassium hydroxide, evaporated *in vacuo* to dryness, and examined in the usual manner for glycerol. The resulting product was a thick syrup that weighed 4.5 gm. It gave no Molisch reaction but a positive test for acrolein. The syrup was presumably crude glycerol, but it was not further identified.

Separation of the Ether-Soluble Constituents—The ethereal solution was dried over sodium sulfate, filtered, and evaporated to dryness. The yellowish residue was dissolved in 400 cc. of ether and diluted with 600 cc. of alcohol, which caused a voluminous, finely divided precipitate to separate. After the mixture had been cooled in ice water the precipitate was filtered off, washed with alcohol, and dried *in vacuo* over sulfuric acid. The slightly yellowish product, consisting mainly of crude mycolic acid, weighed 127.7 gm. This substance was reserved for further investigation.

The filtrate and washings were combined and the ether was removed by distillation, after which an excess of lead acetate dissolved in hot alcohol was added. After the solution had cooled and stood overnight, the lead salts of the fatty acids were filtered off, washed with alcohol, and dried *in vacuo* over sulfuric acid.

The lead salts, on treatment with ether, were separated into ether-soluble and ether-insoluble fractions which were decomposed in the usual manner with dilute hydrochloric acid. The ether-soluble lead salts gave 33.9 gm. of acids as a salve-like mass, while the ether-insoluble lead salts gave 30 gm. of solid fatty acids.

The filtrate and washings from the alcohol-insoluble lead salts were combined and evaporated nearly to dryness *in vacuo*. The residue was treated with 500 cc. of dilute acetic acid and extracted four times with ether. The aqueous portion was then discarded. The ethereal solution was washed with water until the washings were neutral, after which it was extracted three times with 200 cc. portions of dilute sodium hydroxide. The alkaline solutions, on acidification with hydrochloric acid and extraction with ether, yielded 8.9 gm. of liquid fatty acids that had not been precipitated as lead salts.

The ethereal solution, following the alkaline extractions, was washed with water, dried over sodium sulfate, filtered, and evaporated to dryness. The residue consisting of neutral material was a nearly white, partly crystalline mass that weighed 16.6 gm.

Purification of the Carbohydrate—The precipitate that separated from the alkaline benzene solution, as mentioned previously, was dissolved in 200 cc. of water to give a cloudy solution that was strongly alkaline in reaction. The solution was acidified with acetic acid and filtered through a layer of norit. Neutral lead acetate was added to the filtrate and the slight precipitate that separated was filtered off and discarded. The carbohydrate was then precipitated by adding an excess of basic lead acetate and ammonia. The lead salt was filtered off and washed with water, after which it was suspended in water and decomposed with hydrogen sulfide. The lead sulfide was filtered off and the filtrate was concentrated *in vacuo* to a thick syrup. The syrup on dehydration with absolute alcohol gave a white powder that weighed 8.3 gm., $[\alpha]_D$ in H_2O = +51.0°, and there was no mutarotation.

Analysis—Found, N 0 43, P 1.9

The substance gave no reduction with Fehling's solution until after it had been hydrolyzed. On refluxing with 5 per cent sulfuric acid the maximum reduction determined by the Shaffer-Hartmann method (9) was attained in about 3 hours and amounted to 67 per cent, calculated as glucose. The carbohydrate gave the usual pentose color reactions and was apparently similar to the pentose-containing polysaccharide of the human tubercle bacillus.

The various cleavage products of the wax are summarized in Table I.

The balance of this investigation is concerned with a study of the fatty acids obtained from the ether-soluble lead salts, with the solid acids obtained from the ether-insoluble lead salts, and the acids obtained from the alcohol-soluble lead salts, that is, with Fractions 4, 5, and 6 of Table I. Investigations dealing with the crude mycolic acid, Fraction 3, and with the neutral material, Fraction 7, will be reported in subsequent papers.

Fractionation of the Fatty Acids Obtained from the Ether-Soluble Lead Salts. Isolation of Mycocerosic Acid—The fatty acid, 33.9 gm., obtained from the ether-soluble lead salt was saturated, since it showed no iodine number. The acid was converted into the methyl ester by refluxing with absolute methyl alcohol containing 5 per cent of dry hydrochloric acid, after which the ester was isolated in the usual manner and yielded 32.1 gm. of ester as a soft, yellowish mass. The ester was distilled through a modified Widmer column, the apparatus being sealed on to the vacuum line. The system was evacuated by means of a diffusion mercury pump backed by an efficient Hyvac pump, and the pressure was measured on a McLeod gage.

At a pressure of about 10^{-3} mm. the ester distilled at a bath temperature of 230–250° with a vapor temperature of 195–205°. The distillate was almost colorless and it weighed 26.6 gm. The yellowish residue weighed 4.5 gm. Both fractions solidified at room temperature. It is obvious, in view of the high temperature at which the distillate came off, that no lower fatty acids could have been present.

TABLE I

Hydrolysis Products from 227 6 Gm. of Purified Wax

Fraction No	Description	Weight
		gm.
1	Purified carbohydrate	8.3
2	Crude glycerol	4.5
3	" mycolic acid	127.7
4	Fatty acids from ether-soluble lead salts	33.9
5	Solid " " ether-insoluble lead salts	30.0
6	Fatty " " alcohol-soluble " "	8.9
7	Crude neutral material	16.6

Saponification of the Ester—The distilled ester was saponified by refluxing for several hours with an excess of alcoholic potassium hydroxide, after which the free acid was isolated and dried to constant weight *in vacuo* over sulfuric acid. The crude mycocerosic acid formed a non-crystalline waxy solid.

Rotation—0.4597 gm. of acid, dissolved in chloroform and diluted to 10 cc., gave in a 1 dm. tube $\alpha = -0.34°$; hence $[\alpha]_D = -7.39°$.

Titration—0.3695 gm. of acid, dissolved in 25 cc. of neutral alcohol, required 25.51 cc. of 0.01318 N alcoholic potassium hydroxide.

Found, mol. wt. 455
For $C_{30}H_{60}O_2$. Calculated, mol. wt. 452

Fractionation of the Acid—Since Wieghard and Anderson (5) had found that the barium salt of the levorotatory acid that they examined had useful properties, an attempt was made to purify the present acid by the same method. For the preparation of the barium salt the acid was dissolved in

500 cc. of ether and the solution was neutralized to phenolphthalein by the addition of a 10 per cent solution of barium hydroxide in warm methyl alcohol. After the mixture had stood overnight, the granular precipitate was filtered off, washed with methyl alcohol, and dried. The substance was a white powder which weighed 29.0 gm.

The barium salt was dissolved in warm benzene and precipitated by the addition of methyl alcohol. The filtrate from this operation was cooled in ice water, which gave a second fraction. The final filtrate and washings on concentration to dryness gave a third fraction. These operations were repeated on each fraction several times, after which the various fractions were analyzed for barium by combustion to barium carbonate in a platinum crucible. Fractions of similar barium content and solubility were combined, and the free acids were regenerated by treatment in ether suspension with dilute hydrochloric acid. The properties of the acids purified over the barium salt are recorded in Table II.

TABLE II

Properties of Mycocerosic Acid Obtained from the Barium Salts

Fraction No.	Weight	$[\alpha]_D$	n_D^{40}	M p	Mol wt	C	H
	gm			°*C*		*per cent*	*per cent*
1	2.5	−5.10	1.4535	27–28	455	79.56	13.19
2	10.3	−4.03	1.4530	27–29	460	79.76	13.31
3	9.7	−5.07	1.4532	27–27.5	453	79.74	13.27

For $C_{30}H_{60}O_2$. Calculated, C 79.65, H 13.27, mol. wt. 452

It would appear that the values found agree most nearly with the calculated composition of an acid of the formula $C_{30}H_{60}O_2$. As will be noted in Table II, the physical constants of the acid fractions are very similar, thus indicating at least a certain degree of purity. However, when the methyl ester was subjected to fractional distillation, a series of fractions was obtained that varied in properties, thus indicating that the acid was not homogeneous.

A portion of the acid, Fraction 3, Table II, was esterified with diazomethane and 3.88 gm. of the ester were fractionated through a special column.[1] The results are shown in Table III.

The rotations of the first three fractions would indicate an admixture of dextrorotatory acids, such as phthioic acid, whereas the other fractions have more uniform properties.

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[1] This column had been constructed by Dr. S. F. Velick for the purification of the methyl ester of phytomonic acid.

Saponification of the Ester—The ester Fractions 6, 7, 8, and 9 were combined and saponified and the free acid was isolated. The acid was a waxy solid that could not be crystallized. It melted at 27–28°, $[\alpha]_D$ in $CHCl_3$ = −5.7°, mol. wt. by titration 452.8.

$C_{30}H_{60}O_2$. Calculated, mol. wt. 452

The *p*-bromophenacyl ester was prepared according to Judefind and Reid (10). The substance could not be crystallized, but separated as a white amorphous solid from alcohol, m.p. 47–48°.

Analysis—$C_{38}H_{65}O_3Br$ (648.9). Calculated, Br 12.31; found, Br 11.94

The analytical data agree best with the formula $C_{30}H_{60}O_2$ for the mycocerosic acid.

TABLE III

Fractionation of the Methyl Ester of Mycocerosic Acid

Fraction No	Bath temperature	Column temperature	Weight	M p	$[\alpha]_D^{24}$ in $CHCl_3$	d_4^{30}	n_D^{30}
	°C	°C	mg	°C			
1	238	226	65	23	+1.8		1.4418
2	251	245	230	26	−0.4	0.8573	1.4509
3	251	245	525	26	−4.2	0.8636	1.4509
4	255	246	595	25	−6 2	0.8560	1.4502
5	259	248	600	26	−6.9	0.8665	1.4503
6	261	249	655	26	−7.8	0.8544	1.4508
7	269	252	80	24	−6.7		1.4510
8	269	252	215	25	−7.6	0.8541	1.4510
9	278	260	300	25	−7.6	0.8541	1.4509

The Solid Fatty Acids Isolated from the Ether-Insoluble Lead Salts—The solid fatty acids, 30 gm., isolated from the ether-insoluble lead salts, were converted into methyl esters and fractionated through a short modified Widmer column at a pressure of about 0.1 mm. into three fractions and a large residue. The ester fractions were saponified and the free acids were recrystallized from acetone and were obtained as colorless plates. Fraction 1 appeared to be a typical mixture of palmitic and stearic acids. It melted at 57–58° and the molecular weight was 261. Fraction 2 was an apparently similar mixture.

Fraction 3 gave an acid which melted at 63–64°. The molecular weight was 298 and the acid evidently was a mixture of stearic and hexacosanoic acids. No effort was made to purify these acids further, but it seems safe to assume that the principal components were palmitic and stearic acids.

The residue from the distillation after it had been saponified gave an acid fraction that weighed 18.9 gm. It was dissolved in benzene and after standing and cooling it was possible to crystallize 2.5 gm. of an acid which melted at 81–82°. Four recrystallizations from benzene-acetone raised the melting point to 83–84°. The molecular weight by titration was found to be 394.

Analysis—$C_{26}H_{52}O_2$ (396). Calculated, C 78.78, H 13.13
Found, average of three analyses, " 78.99, " 13.09

Although the melting point is somewhat low, the composition of the acid indicates that it is mainly *n*-hexacosanoic acid.

Examination of Mother Liquors after Removing n-Hexacosanoic Acid—The mother liquors on concentration to dryness left a waxy solid acid which weighed 16.3 gm. The acid was levorotatory, $[\alpha]_D$ in $CHCl_3$ = $-4.47°$. The presence of a levorotatory acid in the solid fatty acid fraction was no doubt due to the formation of mixed lead salts with the normal solid fatty acids, thus giving lead salts that were only slightly soluble in ether.

Since the acid could not be crystallized, it was converted into the barium salt, as mentioned previously. The barium salt was separated into several fractions from benzene solution by the gradual addition of methyl alcohol. The main fraction of the acid regenerated from the barium salt was esterified with diazomethane and the ester was fractionated through the special column previously mentioned. The fore run had a low dextrorotation, probably due to admixture of phthioic acid. The next two fractions had low levorotations. The middle fractions corresponded in rotation and physical constants to the purified methyl ester of mycocerosic acid and these fractions were combined and saponified. The free acid corresponded to mycocerosic acid, $[\alpha]_D$ in $CHCl_3$ = $-5.1°$; the molecular weight by titration was 450.

The highest boiling ester fraction gave on saponification a levorotatory acid of very high molecular weight, $[\alpha]_D$ in $CHCl_3$ = $-4.63°$; the molecular weight by titration was 758. The non-volatile residue from the distillation had a levorotation, $[\alpha]_D$ in $CHCl_3$ = $-2.0°$. On saponification it gave an acid which had a molecular weight, by titration, of 1042. These last two fractions were most likely impure mixtures.

Fatty Acids from the Alcohol-Soluble Lead Salts, Fraction 6, Table I—The fatty acid recovered from the alcohol-soluble lead salts weighed 8.9 gm. It was liquid at room temperature and the iodine number was 11. The acid was converted into the lead salt and the latter was treated with ether. The ether-insoluble lead salt gave 1.1 gm. of a white crystalline acid which was not further examined. The acid recovered from the ether-soluble lead salt was reduced with hydrogen and platinum oxide, after which the lead salt-ether separation was repeated. The solid reduced acid obtained from the ether-insoluble lead salt weighed about 1.0 gm. It melted at 54–58° and the molecular weight by titration was 292. This material was not further examined. The acid obtained from the ether-soluble lead salt was esterified by diazomethane and the ester was fractionated repeatedly through the special column, as mentioned previously. The results indicated that the ester was a complex mixture.

The first ester fraction gave on saponification a small amount of a solid fatty acid which was not identified. The next fraction on saponification gave an optically inactive liquid-saturated acid that corresponded in properties and composition to tuberculostearic acid, $C_{19}H_{38}O_2$. The third fraction was dextrorotatory and on saponification gave an acid that had $[\alpha]_D = +5.0°$ and the molecular weight by titration was 397. The fourth fraction was levorotatory and on saponification an acid was obtained that corresponded in properties to mycocerosic acid, $[\alpha]_D$ in $CHCl_3 = -5.1°$, molecular weight by titration 448.

SUMMARY

An examination has been made of the fatty acids contained in the wax isolated from tubercle bacilli residues from the preparation of the purified protein derivative PPD.

The normal fatty acids were represented by palmitic, stearic, and hexacosanoic acids and an unsaturated acid, probably oleic acid.

The branched chain fatty acids giving ether-soluble lead salts were separated into tuberculostearic acid, dextrorotatory acids analogous to phthioic acid, and a levorotatory acid.

The name "mycocerosic acid" is proposed to designate the levorotatory acid which has been found to be a characteristic constituent of all the wax fractions of the human tubercle bacillus.

Mycocerosic acid was obtained as a non-crystalline waxy solid, m.p. 27–28°, $[\alpha]_D$ in chloroform -5 to $-6°$, and its composition corresponds to the formula $C_{30}H_{60}O_2$.

BIBLIOGRAPHY

1. Anderson, R. J., *J. Biol. Chem.*, **83**, 505 (1929).
2. Roberts, E. G., and Anderson, R. J., *J. Biol. Chem.*, **90**, 33 (1931).
3. Stodola, F. H., Lesuk, A., and Anderson, R. J., *J. Biol. Chem.*, **126**, 505 (1938).
4. Anderson, R. J., *J. Biol. Chem.*, **97**, 639 (1932).
5. Wieghard, C. W., and Anderson, R. J., *J. Biol. Chem.*, **126**, 515 (1938).
6. Seibert, F. B., and Glenn, J. T., *Am. Rev. Tuberc.*, **44**, 9 (1941).
7. Creighton, M. M., and Anderson, R. J., *J. Biol. Chem.*, **154**, 581 (1944).
8. Reeves, R. E., and Anderson, R. J., *J. Biol. Chem.*, **119**, 535 (1937).
9. Shaffer, P. A., and Hartmann, A. F., *J. Biol. Chem.*, **45**, 365 (1920–21).
10. Judefind, W. L., and Reid, E. E., *J. Am. Chem. Soc.*, **42**, 1043 (1920).

THE CHEMISTRY OF THE LIPIDS OF TUBERCLE BACILLI

LXXIII. STUDIES ON PHTHIOCEROL*

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In an investigation of the cleavage products of the wax fractions of the human tubercle bacillus, Strain H-37, Stodola and Anderson (1) isolated a new dihydroxymonomethoxy alcohol to which the name "phthiocerol" was assigned. Subsequent work by Reeves and Anderson (2) and Wieghard and Anderson (3) showed that phthiocerol was a constant constituent of all wax fractions obtained from the human tubercle bacillus, and Cason and Anderson (4) found that it was also present in the wax of the bovine tubercle bacillus. Other acid-fast bacteria that have been studied in this laboratory do not contain phthiocerol. The wax fractions of the timothy bacillus (5), the avian tubercle bacillus (6), and the so called leprosy bacillus (7) all contain two higher alcohols, namely *d*-eicosanol-2 and *d*-octadecanol-2, but these alcohols have not been found in the waxes of the human or bovine types of tubercle bacilli. Phthiocerol is undoubtedly identical with an alcohol called "phytoglycol" which was isolated by Stendal (8) from the wax of the human tubercle bacillus, although the formula $C_{26}H_{54}O_2$ was assigned to this substance.

Phthiocerol crystallizes from ethyl acetate in rosettes of prismatic needles, m.p. 72–73°, $[\alpha]_D$ in $CHCl_3 = -4.8°$. It was shown that phthiocerol contains two hydroxyl groups and one methoxyl group, but its constitution has not been determined. It was impossible to decide whether the formula was $C_{34}H_{67}(OH)_2OCH_3$ or $C_{35}H_{69}(OH)_2OCH_3$, but the analytical data favored the first formula.

From a study of the properties of surface films of phthiocerol on different substrates, Ställberg and Stenhagen (9) concluded that it was a very long molecule with only short side chains and with one or more of the polar groups near one end. If side chains other than the methoxyl group were present, they were short, probably methyl.

The parent hydrocarbon of phthiocerol was prepared by Stodola and Anderson (1) by reduction of the iodo compound obtained in the methoxyl

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† Holder of a National Tuberculosis Association Fellowship at Yale University, 1942–44.

determination. The substance, after crystallization from ethyl acetate, melted at 58.5–59.5° and the values found on analysis agreed best with the formula $C_{34}H_{70}$. A sample of the hydrocarbon was furnished to Dr. Stenhagen, who kindly studied its x-ray diffraction pattern in comparison with *n*-tetratriacontane (10). He concluded that it was a normal chain hydrocarbon of the formula $C_{34}H_{70}$, probably *n*-tetratriacontane. However, the specimen sent to Dr. Stenhagen was undoubtedly impure, since on heating it to 130° with concentrated sulfuric acid, considerable darkening occurred.

With the object in view of preparing a larger quantity of the parent hydrocarbon for a more critical purification, the reaction of phthiocerol with hydriodic acid was investigated and the iodo compounds were isolated and analyzed. It was found that prolonged heating of phthiocerol with purified hydriodic acid and phenol was necessary in order to replace the functional groups with iodine. The iodo compounds were rather unstable and after a short time turned yellow with liberation of free iodine. Reduction of the iodo compounds with zinc amalgam in glacial acetic acid resulted in the formation of unsaturated hydrocarbons with iodine numbers from 20 to 32. Catalytic hydrogenation of these hydrocarbons was slow and incomplete. In order to remove the last traces of unsaturated compounds recourse was had to treatment with hot concentrated sulfuric acid. The purified hydrocarbon on recrystallization from ethyl acetate separated in rosettes of prismatic needles similar to phthiocerol itself.

We propose to designate the purified hydrocarbon by the name "phthiocerane." The substance melted at 59–60° and it was optically inactive.

In order to compare phthiocerane with *n*-tetratriacontane a specimen of this hydrocarbon was prepared by electrolysis of stearic acid according to the method of Petersen (11). The hydrocarbon crystallized from ether in large, colorless plates that melted at 72–73° and its crystal form and properties were quite different from those of phthiocerane. It was only slightly soluble in ether or benzene, whereas phthiocerane is very soluble in these solvents.

For further comparison with phthiocerane it seemed of interest to prepare a simply branched hydrocarbon of approximately the same chain length. For this purpose a sample of tuberculostearic acid, which according to Spielman (12) is 10-methylstearic acid, was electrolyzed as described above to yield 9,26-dimethyltetratriacontane,

$$CH_3(CH_2)_7\underset{\displaystyle CH_3}{\underset{|}{CH}}(CH_2)_{16}\underset{\displaystyle CH_3}{\underset{|}{CH}}(CH_2)_7CH_3$$

This hydrocarbon was purified by distillation *in vacuo* and was obtained as an oil at room temperature but which solidified between 15–20°, and it was very soluble in ether. It is interesting that the presence of two methyl groups on a hydrocarbon chain of 34 carbon atoms gives rise to a product of very low melting point.

EXPERIMENTAL

Purification of Phthiocerol—The phthiocerol used in the present investigation was isolated from the crude neutral material resulting from the saponification of 227.5 gm. of purified, chloroform-soluble wax from cell residues remaining from the preparation of a special lot of purified protein derivative PPD, as described in Paper LXXII of this series (13). The substance was purified by several crystallizations from ethyl acetate and separated in colorless aggregates of fine prismatic crystals. The purified phthiocerol weighed 12.1 gm. and, in agreement with previous reports, the substance melted at 72–73°, $[\alpha]_D$ in $CHCl_3$ was −4.37°.

Analysis—Mol. wt., Rast, found, 550, 539
Hydroxyl value, " 5 91
Methoxyl " " 5.44, 5 36
$C_{35}H_{72}O_3$ (540) Calculated, 2OH 6 29, OCH_3 5.74
$C_{36}H_{74}O_3$ (544). " " 6 13, " 5.59

It is evident from the properties that the substance is identical with the phthiocerol previously described.

In order to test the homogeneity of the preparation, about 100 mg. of the substance were heated in a Washburn (14) molecular still to 160°. In the course of 3 hours about 10 mg. of distillate were collected. Both the distillate and the residue melted at 72–73° and a mixed melting point was also 72–73°. Since no fractionation occurred, the substance was evidently homogeneous.

Phthiocerol does not give any satisfactory derivatives that may be used for identification purposes. The acetyl and benzoyl derivatives are noncrystalline. The phenylurethane (4), of the several urethanes that have been prepared, had the most promising properties but it was amorphous and melted between 78–82°. In the present study the 3,5-dinitrobenzoyl derivative was prepared, but it was an oil at room temperature. Another attempt was made to prepare the phenylurethane, but the yield was poor. From 0.5 gm. of phthiocerol, on treatment with phenyl isocyanate, it was possible after several precipitations from methyl alcohol to isolate 140 mg. of small, rod-shaped crystals that melted at 91–92°.

Analysis—$C_{49}H_{82}O_5N_2$ (778). Calculated, C 75.58, H 10.54, N 3.59
Found, " 75.99, " 10.57, " 3.56

For the purpose of identification we have found that phthiocerol itself is more easily purified and identified by its properties than is the case with any derivatives so far examined.

Reactions of Phthiocerol with Hydriodic Acid—It was hoped that the preparation and analysis of the triiodo compound formed by replacement of the three functional groups by iodine would serve to distinguish between the two formulas suggested for phthiocerol, since there is an appreciable difference in iodine content; namely $C_{34}H_{67}I_3$ requires 44.51 per cent and $C_{35}H_{69}I_3$ requires 43.79 per cent of iodine. This hope was not realized because no pure triiodo compound could be obtained.

The reaction was carried out by refluxing for varying periods of time in an atmosphere of nitrogen 1.0 gm. of phthiocerol with 1.0 gm. of phenol and 15 cc. of hydriodic acid, sp. gr. 1.7, which had been freshly distilled over red phosphorus. After the reaction mixture had cooled, it was diluted with water and extracted with ether. The ethereal solution was washed thoroughly with sodium bicarbonate solution, followed by washing with a solution of sodium thiosulfate and with water. The ethereal solution was then dried over sodium sulfate, filtered, and evaporated to dryness. The residue was dissolved in ether and precipitated by the addition of cold methyl alcohol. The precipitations were repeated until a white granular product was obtained. The iodo compounds gradually assumed a yellow color on standing and the odor of iodine became noticeable.

When the reaction mixtures were refluxed for 2 or 3 hours, the isolated iodo compounds contained from 22 to 36 per cent of iodine. When the refluxing was continued for 34 hours, the iodine content of the iodo compounds varied from 43 to 59 per cent. The results indicated that prolonged refluxing with hydriodic acid was necessary to replace completely the functional groups with iodine. It was also evident that in some cases substitution of iodine had occurred.

Preparation of Phthiocerane—For the preparation of the hydrocarbon phthiocerol was refluxed with hydriodic acid and phenol, as mentioned above, for 34 hours. The iodo compounds were isolated and reduced by refluxing for 5 hours with 25 cc. of benzene, 10 cc. of glacial acetic acid, and an excess of zinc amalgam, after which the solvents were removed by distillation *in vacuo*. The residue was mixed with water and extracted with ether. The ethereal solution was washed with dilute sodium bicarbonate solution and with water, dried over sodium sulfate, filtered, and evaporated to dryness. The residues were white, wax-like solids that were free of halogen, but they were unsaturated, showing iodine numbers ranging from 20 to 32.

The unsaturated hydrocarbons obtained in these reactions could not be reduced completely with hydrogen and platinum oxide. In one case a preparation having an original iodine number of 24 was subjected to catalytic hydrogenation in the presence of active platinum oxide in a Parr bomb for a period of 50 hours, but the substance still had an iodine number of 2.4.

In order to remove the last traces of unsaturated compounds, the hydrocarbon was heated with concentrated sulfuric acid to 120°, whereupon a deep red color developed. The reaction mixture was cooled, diluted with water, and extracted with ether. The ethereal solution was washed with water, dried over sodium sulfate, decolorized with norit, filtered, and evaporated to dryness. The residue was recrystallized from ethyl acetate and was obtained in the form of rosettes of fine needles, quite similar in appearance to phthiocerol itself. No color developed on reheating to 120° with concentrated sulfuric acid. The hydrocarbon was free from sulfur and halogen. In chloroform solution it showed no optical activity.

The purified phthiocerane melted at 59–60°.

Analysis—$C_{34}H_{70}$ (478).	Calculated,	C 85 35,	H 14.64
	Found,	" 85.34, 85.48,	" 14 64, 14.60

Phthiocerane behaved like a homogeneous substance when subjected to molecular distillation. Both the distillate and the residue had the same melting point and also the same mixed melting point. Phthiocerane was very soluble in ether and in benzene. Its low melting point and high solubility in ether would indicate that it is not a normal straight chain hydrocarbon.

In order to compare the properties of other hydrocarbons containing the same number of carbon atoms in the chain as phthiocerane, we synthesized *n*-tetratriacontane and the branched chain hydrocarbon 9,26-dimethyltetratriacontane. The properties of both of these hydrocarbons were different from those of phthiocerane.

Synthesis of n-Tetratriacontane—The hydrocarbon was synthesized by electrolysis of 10 gm. of stearic acid, as described by Petersen (11). The reaction product which separated as an oil solidified at room temperature and was collected, washed with water, and was then refluxed with alcoholic potassium hydroxide for 1 hour. After the solution had cooled, it was diluted with water, and the precipitate that separated was filtered off, washed thoroughly with water, and dried *in vacuo*. The substance, after it had been crystallized from benzene, was dissolved in 350 cc. of boiling ether. As the solution cooled large, colorless, plate-shaped crystals separated which were filtered off, washed with ether, and dried. The crystals weighed 3.5 gm. and the melting point was 72–73°, in agreement with the recorded melting point of *n*-tetratriacontane.

In view of the great differences in solubility and in melting points of phthiocerane and *n*-tetratriacontane, we do not believe that the two hydrocarbons are identical.

Synthesis of 9,26-Dimethyltetratriacontane—For the preparation of 9,26-dimethyltetratriacontane 5 gm. of tuberculostearic acid were electrolyzed according to Petersen (11) and the reaction product was isolated as described for *n*-tetratriacontane. The hydrocarbon was an oil at room temperature and it was very soluble in ether and benzene. It was purified by distillation through the column developed by Dr. S. F. Velick of this laboratory. At a pressure of about 1 mm. the hydrocarbon distilled as a colorless oil at a bath temperature of 264° and a column temperature of 253°. The yield of the purified hydrocarbon was 0.7 gm. On cooling the oil solidified between 15–20°. The following constants were determined:

n_D^{25} 1.4549, d_4^{25} 0.8128

Analysis—$C_{36}H_{74}$(506). Calculated, C 85 37, H 14.62
Found, " 85.24, " 14.69

DISCUSSION

The results reported in this paper show that the alcohol occurring in the wax obtained from cell residues from the preparation of tuberculin is identical with the alcohol named "phthiocerol" which we have formerly isolated from the wax fractions of Strain H-37, as well as from other strains of the human tubercle bacillus. Phthiocerol crystallizes readily from ethyl acetate or acetone and can be identified by its properties, but it does not yield satisfactory derivatives. So far it has not been possible to determine whether its formula is $C_{34}H_{67}(OH)_2OCH_3$ or $C_{35}H_{69}(OH)_2OCH_3$ but the analytical data favor the first formula.

The reaction of phthiocerol with hydriodic acid is rather complicated. The methoxyl group is split off very readily in the Zeisel determination, but the replacement of the hydroxyl groups with iodine requires prolonged refluxing with hydriodic acid and does not yield very satisfactory iodo derivatives. The reduction of the iodo derivatives is easily accomplished, but the resulting hydrocarbons are unsaturated and are difficult to hydrogenate.

The parent hydrocarbon of phthiocerol, which has been named "phthiocerane," crystallizes from ethyl acetate and is undoubtedly homogeneous, and its composition corresponds to the formula $C_{34}H_{70}$ or possibly $C_{35}H_{72}$. The low melting point of phthiocerane, which lies 13° below that of *n*-tetratriacontane, and its high solubility in ether and benzene would indicate that it is not a normal straight chain hydrocarbon.

It is very probable that phthiocerol and phthiocerane contain a long straight carbon chain, as suggested by Stenhagen, with a simple side chain, possibly a methyl or ethyl group. It is hoped that further x-ray studies on the purified phthiocerane will shed more light upon its structure.

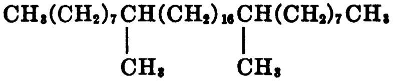
SUMMARY

1. Evidence is presented which indicates that phthiocerol is a homogeneous substance and that its formula is either $C_{34}H_{67}(OH)_2OCH_3$ or $C_{35}H_{69}(OH)_2OCH_3$.

2. The reaction of phthiocerol with hydriodic acid has been studied and the resulting iodo derivatives, which are rather unstable, have been analyzed.

3. Phthiocerane, the hydrocarbon prepared from phthiocerol by reduction of the iodo derivative, has been obtained in very pure form. It crystallizes in rosettes of fine needles and melts at 59–60°. Its formula is either $C_{34}H_{70}$ or $C_{35}H_{72}$. Its low melting point and high solubility would indicate that it has a branched chain structure.

4. A new branched chain hydrocarbon, 9,26-dimethyltetratriacontane,



$$CH_3(CH_2)_7\underset{\displaystyle CH_3}{\underset{|}{CH}}(CH_2)_{16}\underset{\displaystyle CH_3}{\underset{|}{CH}}(CH_2)_7CH_3$$

has been synthesized by electrolysis of tuberculostearic acid and some of its properties have been determined.

BIBLIOGRAPHY

1. Stodola, F. H., and Anderson, R. J., *J. Biol. Chem.*, **114**, 467 (1936).
2. Reeves, R. E., and Anderson, R. J., *J. Biol. Chem.*, **119**, 535 (1937).
3. Wieghard, C. W., and Anderson, R. J., *J. Biol. Chem.*, **126**, 515 (1938).
4. Cason, J., and Anderson, R. J., *J. Biol. Chem.*, **119**, 549 (1937).
5. Pangborn, M. C., and Anderson, R. J., *J. Am. Chem. Soc.*, **58**, 10 (1936).
6. Reeves, R. E., and Anderson, R. J., *J. Am. Chem. Soc.*, **59**, 858 (1937).
7. Anderson, R. J., Crowder, J. A., Newman, M. S., and Stodola, F. H., *J. Biol. Chem.*, **113**, 637 (1936).
8. Stendal, N., *Compt. rend. Acad.*, **198**, 1549 (1934).
9. Ställberg, S., and Stenhagen, E., *J. Biol. Chem.*, **143**, 171 (1942).
10. Stenhagen, E., *J. Biol. Chem.*, **148**, 695 (1943).
11. Petersen, J., *Z. Elektrochem.*, **12**, 141 (1906).
12. Spielman, M. A., *J. Biol. Chem.*, **106**, 87 (1934).
13. Ginger, L. G., and Anderson, R. J., *J. Biol. Chem.*, **157**, 203 (1945).
14. Washburn, E. W., *Bur. Standards J. Res.*, **2**, 476 (1929).

STUDIES ON BIOLOGICAL OXIDATIONS

XIX. SULFHYDRYL ENZYMES IN CARBOHYDRATE METABOLISM

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During the last 12 years, the chemical composition of enzymes has been, to a certain extent, elucidated. It is now recognized that most of them are complex systems made up of a number of components which might be separated as individual molecular entities. Of these components, the protein moiety determines the specificity of the enzyme system; the other components can be replaced by substances of similar properties. A knowledge of the properties of these proteins, of the chemical groups essential for enzymatic activity, and of the nature of enzyme-substrate combination is therefore of fundamental importance. The protein moiety of the enzyme may be of the fibrous or of the corpuscular type; a small alteration in the spatial configuration of the protein or a small chemical change in some of the groups of the side chains might be enough to destroy enzyme activity. This is indication that there exist in the protein molecule certain bonds and certain groups, located in the side chains, without whose integrity no enzyme activity exists. Of these groups, the electronegative —SH groups have received most attention because of the ease and reliability of their detection. Of the numerous investigators who have devoted their attention to this subject, Hellerman and his coworkers, Hopkins and his coworkers, and Rapkine have made the most illuminating contribution and have inspired other workers in the search for these —SH groups among the proteins of enzyme systems. Indeed, the discovery by Hellerman *et al.* (1) of the existence in urease of sulfhydryl groups which are easily available and not necessary for enzyme activity, and others less readily available and essential for enzymatic activity, has greatly clarified many seemingly contradictory reports. The discovery by Hopkins *et al.* (2), that previous addition of malonate or succinate protects succinoxidase from inhibition by —SH inhibitors, and that of Rapkine (3), that previous addition of diphosphopyridine nucleotide protects phosphoglyceraldehyde oxidase from inhibition, have given indications for considering those —SH groups as places where union between protein and substrate or protein and prosthetic group occurs.

Up to 1938, sulfhydryl enzymes were reported only among certain hydrolytic enzymes (see the reviews by Bersin (4) and by Hellerman (5)).

The presence of sulfhydryl groups in oxidation enzymes was first demonstrated in 1938 by Hopkins and Morgan (6) in succinoxidase and by Rapkine (3) in phosphoglyceraldehyde oxidase. These findings added to those suggesting the presence of essential —SH groups in phosphoglucomutase (Lehmann (7)), hexose monophosphate oxidase (von Euler and Adler (8)), yeast alcohol oxidase (Wagner-Jauregg and Möller (9), Dixon (10)), and glycerol oxidase (Barron (11)) imply a wide distribution of the —SH enzymes (enzymes requiring the presence of certain —SH groups for enzymatic activity). In our search for the active groups of proteins responsible for enzyme activity, it was decided to start with a study of the —SH groups essential for enzymatic activity in those processes concerned with the breakdown and the synthesis of carbohydrates.

EXPERIMENTAL

Reagents for Detection of —SH Group in Enzymes—For the detection of —SH groups in enzymes use has been made of some oxidizing agents, alkylating agents, and mercaptide-forming compounds. However, since the sulfhydryl groups of proteins show different degrees of affinity for these compounds, it is preferable to submit the enzymes to the action of the three kinds of reagents before coming to the conclusion that no —SH groups are necessary for enzyme activity. The danger of using single reagents, especially those that act by oxidation, has been sufficiently emphasized by Anson (12). Three types of substances were used as a rule in our studies: iodoacetamide, *p*-chloromercuribenzoate, and trivalent organic arsenicals. As the last two reagents combine reversibly with the —SH groups, reactivation of the inactivated enzymes was attempted with either cysteine or glutathione (GSH). On some occasions alloxan, porphyrindine, iodosobenzoate, and maleate were used. The oxidation of —SH groups of proteins by alloxan was demonstrated by Labes and Freisburger (13). The use of maleate as —SH reagent was introduced by Morgan and Friedmann (14). Hellerman *et al.* (15) introduced *p*-chloromercuribenzoic acid and *o*-iodosobenzoate as —SH reagents. Iodoacetamide and porphyrindine were prepared at the laboratory; *p*-chloromercuribenzoic acid and the organic arsenicals were generously provided by Dr. L. Hellerman and by Dr. Harry Eagle. Glutathione was obtained from Schwartz Laboratories, Inc., New York.

Methods

Pyruvate was determined either manometrically with yeast carboxylase or colorimetrically by a modification of the method of Lu (16). α-Ketoglutarate was determined colorimetrically as in Lu's method with the exception that the wave-length used was 5200 A, instead of 4200 A as in pyruvate. Phosphorus was determined colorimetrically by the method of Berenblum and Chain (17) at a wave-length of 7300 A. Diphosphopyridine nucleotide was determined at a wave-length of 3450 A. All these determinations were carried out with the Beckman photoelectric quartz spectrophotometer. Succinate was determined, after continuous extraction with ether for 4 hours, by its oxidation with pigeon breast succinoxidase. α-Ketoglutarate was determined according to Krebs (18) by oxidizing it to succinate with permanganate and subsequent extraction of the succinate. Acetoacetate was measured manometrically with aniline citrate (Edson (19)).

Succinoxidase—In 1938, Hopkins and Morgan demonstrated the presence of essential —SH groups in the activating protein (dehydrogenase) of succinoxidase. The same authors, in collaboration with Lutwak-Mann (2) found that succinate and malonate protected the —SH groups of the enzyme from —SH inhibitors. Succinoxidase was chosen for a comparative study of the inhibiting effects of a representative group of —SH reagents.

Succinoxidase was prepared as follows: Pigeon breast muscle cut in small pieces was put into a Waring blendor containing 200 cc. of distilled water and an equal volume of crushed ice. After being stirred at high speed for 3 minutes, it was centrifuged for 30 minutes at 2500 R.P.M. and the supernatant fluid was discarded; this washing was repeated. The solid mass was shaken with an equal volume of 0.1 M Na_2HPO_4 for 3 hours at 30° and after centrifugation at 2500 R.P.M. for 30 minutes the supernatant fluid was used. The QO_2 of such a preparation was around 350 at pH 7.4 and 38°. It contained no lactic or malic dehydrogenases. In Table I are given the relative degrees of inhibition of succinoxidase by arsenicals, *p*-chloromercuribenzoate, arsenite, iodoacetamide, iodoacetate, and three oxidizing agents used for the determination of —SH groups in proteins; namely, ferricyanide, porphyrindine, and *o*-iodosobenzoate. The arsenicals and *p*-chloromercuribenzoate had about the same combining power for the —SH groups of the protein, as is clearly demonstrated in Fig. 1, where the degree of inhibition was plotted against the concentration of the inhibitors. 50 per cent inactivation was obtained with 3.2×10^{-5} M *p*-chloromercuribenzoate and with 3.15×10^{-5} M 3-amino-4-hydroxyphenyldichloroarsine hydrochloride. The affinity of these compounds for the —SH groups of the enzyme was much greater than the affinity of the enzyme for succinate and for malonate (with succinate concentration at 5×10^{-2} M). With increasing concentrations of succinate, 50 per cent activity was reached with 200×10^{-5} M. In the presence of 0.05 M succinate, 125×10^{-5} M malonate produced 50 per cent inhibition.

Of the oxidizing agents used (at equimolecular concentrations) *o*-iodosobenzoate was the most powerful inhibitor, porphyrindine came next, while ferricyanide had a low inhibiting action at this concentration. This explains why ferricyanide could be used by Quastel and Wheatley (20) as the oxidizing agent of succinate when its oxidation by succinoxidase was performed in absence of oxygen.

Of the alkylating agents, iodoacetate has been extensively used, although Michaelis and Schubert (21) called attention to the combination of acid halides with —NH_2 groups. This possibility is usually dismissed as occurring in enzyme inhibitions, because it is assumed that in neutral solutions iodoacetate combines only with —SH groups. If this assumption is correct, once all the —SH groups necessary for activity are covered, say by mercaptide formation, iodoacetate ought to have no action on the enzyme. Addition of a thiol in concentration sufficient to combine with iodoacetate and to dissociate the mercaptide ought to produce reactivation of the enzyme to the same extent as reactivation after inhibition with the mercaptide-forming compound above. (GSH does not reverse the combination of the protein with iodoacetate.)

TABLE I

Effect of —SH Reagents on Succinoxidase

Enzyme, from pigeon breast muscle; pH 7.4 (0.033 M PO_4); succinate, 0.02 M; 38°; duration of experiments, 20 minutes; QO_2 of a typical preparation, 351 c.mm. For convenience the arsenicals are called by their numbers in the text.

Inhibitor	Concentration	Inhibition
	M	*per cent*
p-Carboxyphenylarsine oxide (I)	$4\ 2 \times 10^{-5}$	79.3
p-Carbamylphenylarsine " (II)	4.2×10^{-5}	80
p-Aminophenyldichloroarsine hydrochloride (III)	4.2×10^{-5}	80.5
3-Amino-4-hydroxyphenyldichloroarsine hydrochloride (IV)	4.2×10^{-5}	51.3
Phenylarsine oxide (V)	4.2×10^{-5}	78.1
Sodium arsenite	5×10^{-3}	15
Iodoacetamide	5×10^{-3}	64
p-Chloromercuribenzoate	5×10^{-5}	73.5
Iodoacetate	1×10^{-3}	42
Ferricyanide	5×10^{-4}	28
o-Iodosobenzoate	5×10^{-4}	Complete
Porphyrindine	5×10^{-4}	90

To test this assumption, samples of succinoxidase were inhibited by *p*-chloromercuribenzoate to complete inhibition, by iodoacetate to 86 per cent inhibition, and by iodoacetamide to 80 per cent inhibition. To other vessels containing enzyme plus *p*-chloromercuribenzoate there were added 10 minutes later iodoacetate and iodoacetamide. At the end of 35 minutes the reactivation of the enzyme was performed with glutathione. The enzyme inhibited by *p*-chloromercuribenzoate alone was 84 per cent reactivated by glutathione; in the presence of *p*-chloromercuribenzoate plus iodoacetate, there was only 13 per cent reactivation; in the presence of iodoacetamide plus *p*-chloromercuribenzoate the reactivation reached 68 per cent. These experiments show that iodoacetate and iodoacetamide may produce enzyme inhibition by a mechanism other than combination with —SH groups, the first to a large extent, the second to a smaller degree (Table II).

Hopkins *et al.* (2) on reporting the inhibition produced by oxidized glutathione emphasized that the inhibition was a slow process reaching its maximum intensity gradually. Such was not the case in the inhibition by arsenicals, in which maximum inhibition was attained 6 minutes after addition of the arsenical. While the oxidation of —SH groups was a slow process, the formation of thioarsenite was rather quick.

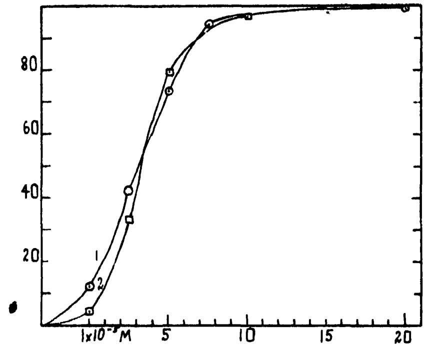


FIG. 1. Inhibition of succinoxidase by *p*-chloromercuribenzoate and organic arsenicals. Abscissa, concentration of inhibitor, × 10^{-5} M; ordinate, per cent inhibition. Curve 1, *p*-chloromercuribenzoate; Curve 2, 3-amino-4-hydroxyphenyldichloroarsine hydrochloride. 1 cc. of enzyme; 1 cc. of phosphate buffer, 0.066 M, pH 7.2; 0.25 cc. of 0.25 M succinate; total volume, 2.5 cc.

The very interesting observation of Hopkins *et al.* (2) on the protection by malonate against inhibition by oxidized glutathione has also been noted when arsenicals were used as inhibitors. 7.5 × 10^{-5} M Arsenical IV produced in these experiments 73 per cent inhibition when added before the succinate; 2.5 × 10^{-3} M malonate produced an inhibition of 43 per cent. When malonate and arsenical were added together, the O_2 uptake was almost the same as when only malonate was present (Table III). Potter and DuBois (22) have also repeated these experiments of Hopkins *et al.*, using quinone as inhibitor, and have confirmed this protective action of malonate.

Enzymes for Pyruvate Metabolism—It is now recognized that pyruvate, the end-product of the first phase of carbohydrate fermentation (23), is extremely reactive and undergoes decarboxylation, oxidation, dismutation, condensation, and CO_2 fixation. Whether all these reactions are catalyzed by the same enzyme or by different enzymes has not yet been established, although there is more evidence in favor of the second assumption. The search for —SH groups among these enzyme systems was made by studying the different reactions in which pyruvate is one of the reactants under the optimum conditions for each reaction. The —SH nature of pyruvate oxi-

TABLE II

Effect of Iodoacetate and Iodoacetamide on Succinoxidase Treated with p-Chloromercuribenzoate. Reactivation by Glutathione

p-Chloromercuribenzoate, 5×10^{-4} M; CH_2ICOOH, 3×10^{-2} M; $CH_2ICOONH_2$, 5×10^{-3} M; succinate 5×10^{-2} M; buffer, phosphate, 0.03 M, pH 7.2. Succinoxidase treated with those reagents either alone or in combination for 35 minutes at 23°. Enzyme activity tested at 38°.

System	O_2 uptake in 30 min.	Inhibition	Reactivation
	c.mm.	*per cent*	*per cent*
Control	395		
p-Chloromercuribenzoate	0	Complete	
CH_2ICOOH	54	86	
$CH_2ICOONH_2$	81	80	
p-Chloromercuribenzoate + CH_2ICOOH	0	Complete	
“ + $CH_2ICOONH_2$	0	“	
“ + GSH, 0.01 M	333.5	15.5	84.5
“ + CH_2ICOOH + GSH, 5.3×10^{-2} M	51	87	13
p-Chloromercuribenzoate + $CH_2ICOONH_2$ + GSH, 4.7×10^{-2} M	250	32	68.4

TABLE III

Protective Action of Malonate against Arsenical Inhibition of Succinoxidase (Hopkins Phenomenon)

System	O_2 uptake in 20 min.	Inhibition
	c.mm.	*per cent*
Succinate, 0.05 M	318.7	
“ and 7.5×10^{-5} M Arsenical IV	85.5	73.2
“ “ 2.5×10^{-3} “ malonate	179.6	43.7
“ “ malonate (2.5×10^{-3} M) and Arsenical IV (7.5×10^{-5} M)	169.7	46.7

dase was first suggested by Peters (24), and some evidence in favor of this suggestion is found in Barron's paper on α-ketonoxidase from gonococci (25). Pyruvate oxidation was found quite sensitive to atmospheric oxygen, and was inhibited by quinone at low concentrations (90 per cent inhibition with 3×10^{-5} M quinone). Snell and Weissberger (26) 3 years later presented evidence for the formation of addition compounds between quinone and thiols.

The effect of sulfhydryl reagents on *pyruvate oxidation* was studied in the enzyme present in gonococci, in ground, washed avian liver, and in rat liver slices. In the first two, O_2 uptake was measured (gonococci take up no oxygen in the absence of pyruvate and the residual oxygen uptake of washed liver is small); in rat liver, the pyruvate utilization was determined. The oxidation of pyruvate by gonococci was inhibited completely by Arsenicals IV and I; the oxidation by ground chicken liver (prepared as described by Barron *et al.* (27)) was inhibited by Arsenical I, *p*-chloromercuribenzoate, and iodoacetamide. The utilization of pyruvate by rat liver slices was inhibited by Arsenical III (75 per cent) (Table IV).

TABLE IV

Effect of —SH Reagents on Pyruvate Oxidation

Experiment I, gonococci suspension in Ringer-PO_4 solution; pH 7.4; pyruvate concentration, 0.02 M; gas phase, air; 38°. Experiment II, Ringer-PO_4 solution; pH 7.4; Mg^{++}, Mn^{++}, and diphosphothiamine added; pyruvate concentration, 0.02 M; gas phase, air; 38°. Experiment III, Ringer-PO_4 solution; pH 7.4; gas phase, O_2; 38°.

Experiment No.	Source of enzyme	Inhibitor	Concentration	O_2 uptake or pyruvate utilization per mg	Inhibition
			M	c.mm.	per cent
I	Gonococci (O_2 uptake)	None		298	
		Arsenical IV	1×10^{-4}	18	94
		" I	1.1×10^{-4}	0	Complete
II	Washed, ground chicken liver (O_2 uptake blank subtracted)	None		220.5	
		Arsenical I	1×10^{-4}	0	Complete
		p-Chloromercuribenzoate	5×10^{-4}	17.2	92
		Iodoacetamide	5×10^{-3}	0	Complete
III	Rat liver slices (pyruvate utilization)	None		72.6	
		Arsenical III	5×10^{-4}	18.1	75

The dismutation of pyruvate ($2CH_3COCOOH \rightarrow CH_3CHOHCOOH + CH_3COOH + CO_2$) was studied in minced pigeon liver suspended in Ringer-bicarbonate solution, with N_2-CO_2 as the gas phase. The CO_2 formation and pyruvate utilization were determined manometrically. The inhibiting effect of the —SH reagents was not as marked as in pyruvate oxidation. In fact , concentrations of arsenicals, *p*-chloromercuribenzoate, and iodoacetamide which produced complete inhibition of pyruvate oxidation gave only partial inhibition of pyruvate dismutation. However, 1×10^{-3} M *p*-chloromercuribenzoate and 5×10^{-3} M iodoacetamide inhibited this reaction completely. *The formation of acetoacetate* from pyruvate was studied in ground pigeon liver in the presence of malonate (1×10^{-2} M). Arsenical III (2×10^{-3} M), *p*-chloromercuribenzoate (1×10^{-3} M), and iodoacetamide (5×10^{-3} M) inhibited completely this pyruvate condensation reaction. *The formation of acetylmethylcarbinol* was studied with the enzyme prepared from heart according to Green *et al.* (28), by determining the CO_2 formation manometrically in the presence of pyruvate and acetaldehyde, in N_2-CO_2 as gas phase. This reaction was inhibited 46 per cent by 1×10^{-3} M Arsenical IV, 85 per cent by 1×10^{-3} M *p*-chloromercuribenzoate, and 64 per cent by 5×10^{-3} M iodoacetamide. *The formation of α-ketoglutarate* (CO_2 fixation?) was studied with pigeon liver extract, prepared according to Evans and Slotin (29). The enzyme, extracted with NaCl-bicarbonate saturated with O_2-CO_2 (pH 7.4), was incubated with 5×10^{-2} M pyruvate and 6.6×10^{-3} M malonate (O_2-CO_2 as gas phase). Arsenical I (5×10^{-4} M) inhibited the reaction 73 per cent; iodoacetamide (5×10^{-3} M), 74 per cent; maleate (2.5×10^{-3} M), 26 per cent.

J. Meyer, of this laboratory, found (unpublished experiments) that *yeast carboxylase* was inhibited by the —SH reagents iodoacetate, iodoacetamide, iodine, $CuCl_2$, *p*-chloromercuribenzoate. He observed, furthermore, that the extent of inhibition was greater when the —SH reagent was added to carboxylase in the absence of cocarboxylase (alkaline washed yeast), an indication that the prosthetic group was attached to the protein at the side chains of the —SH groups, and in agreement with Rapkine's observation on phosphoglyceraldehyde oxidase and protection with pyridine nucleotide. Further evidence that carboxylase is an —SH enzyme was obtained by inhibition with Arsenical III (Table V). Greenberg and Rinehart (30) offered indirect evidence of the necessity of —SH groups for enzyme activity of carboxylase in experiments in which the activity of alkaline washed yeast was increased on addition of thiol compounds (glutathione and cysteine).

α-Ketoglutarate Oxidase—Krebs and Johnson (31) found that in the presence of arsenite minced pigeon breast muscle converts quantitatively citric acid into α-ketoglutaric acid. The experiments presented in Table VI show that this inhibition of α-ketoglutarate oxidation by arsenite (in the absence of arsenite, α-ketoglutarate is oxidized by minced pigeon breast muscle) is due to a combination with essential —SH groups of the activating protein of the enzyme. The oxidation of α-ketoglutarate by kidney slices in the

TABLE V

Effect of —SH Reagents on Enzyme Systems for Pyruvate Metabolism

Experiment I, ground, washed pigeon liver; buffer, NaCl-$NaHCO_3$, pH 7.4; pyruvate, 0.02 M (Mn^{++}, Mg^{++}, and diphosphothiamine added); gas phase, N_2-CO_2; 38°. Experiment II, ground liver suspended in NaCl-phosphate, pH 7.4; malonate, 0.02 M; pyruvate, 0.02 M; air as gas phase; 38°. Experiment III, enzyme from pig heart suspended in phosphate-bicarbonate, pH 6.0; gas phase, N_2-CO_2; 38°. Experiment IV, enzyme extracted from pigeon liver, suspended in Ca-free bicarbonate-Ringer's solution, pH 7.4; pyruvate, 0.04 M; malonate, 0.066 M; gas phase, O_2-CO_2; 38°. Experiment V, washed brewers' yeast, 30 mg. per vessel; acetate buffer, pH 4.81; 25°.

Experiment No.	Enzyme system	Inhibitor	Concentration	Reaction expressed as c.mm. O_2 uptake, pyruvate utilization, or formation of acetoacetate and α-ketoglutarate	Inhibition
			M		per cent
I	Pyruvate dismutation (anaerobic pyruvate utilization)	None		14.1	
		Arsenical III	5×10^{-4}	9.1	35.5
		p-Chloromercuribenzoate	5×10^{-4}	10.0	29.0
		Iodoacetamide	5×10^{-4}	12.1	14.2
		p-Chloromercuribenzoate	1×10^{-3}	0	Complete
		Iodoacetamide	5×10^{-3}	0	"
II	Pyruvate condensation (acetoacetate formation)	None		147.4	
		Arsenical III	2×10^{-3}	0	Complete
		p-Chloromercuribenzoate	1×10^{-3}	0	"
		Iodoacetamide	5×10^{-3}	0	"
III	Pyruvate condensation (acetylmethylcarbinol formation) determined by CO_2 release	None		230.6	
		Arsenical IV	1×10^{-3}	124.2	46
		p-Chloromercuribenzoate	1×10^{-3}	33.7	85.4
		Iodoacetamide	5×10^{-3}	82.0	64.5
IV	Pyruvate CO_2 fixation (α-ketoglutarate formation)	None		1785	
		Arsenical	5×10^{-4}	478	73.2
		Maleate	2.5×10^{-3}	1320	26.0
		Iodoacetamide	5×10^{-3}	460	74.0
V	Pyruvate decarboxylation	None		115	
		p-Chloromercuribenzoate	1×10^{-3}	0	Complete
	Yeast carboxylase* (CO_2 formation)*	Iodoacetamide	5×10^{-3}	31	73
		Iodoacetate	5×10^{-3}	46	60
		None		208	
		Arsenical III	1.2×10^{-3}	21.8	89

* Values obtained by Mr. J. Meyer in alkaline washed yeast.

presence of malonate was inhibited by the three most specific reagents for —SH group detection, organic arsenical, *p*-chloromercuribenzoate, and iodoacetamide.

Malate Oxidase—Green (32) in his paper on malic dehydrogenase reports that 38 per cent of the activity of malate oxidase was lost on treatment with 0.03 M iodoacetate, and that 0.03 M arsenite ("arsenious acid") and 0.03 M maleate had no effect. Evidence that the activating protein of this enzyme belongs to the group of —SH enzymes is given in Table VII. The activating protein (dehydrogenase) of malate oxidase, prepared according to Green, was treated with organic arsenical, *p*-chloromercuribenzoate, and iodoacetamide, enzyme activity being measured by the O_2 uptake in the presence of diphosphopyridine nucleotide and semicarbazide. (Sufficient amounts of alloxazine dinucleotide and cytochrome-cytochrome oxidase exist in the enzyme suspension to allow a steady O_2 uptake.) *p*-Chloromercuribenzoate at a concentration of 2×10^{-4} M inhibited completely the O_2 uptake; the same concentration of Arsenical V produced an inhibition of 51 per cent, while 3×10^{-3} M iodoacetamide had no effect at all. The —SH groups of this enzyme, so readily destroyed by Hellerman's reagent, resisted somewhat the action of arsenicals and were not acted upon by iodoacetamide (Table VII).

TABLE VI

Inhibition of α-Ketoglutarate Oxidase by —SH Reagents

Rat kidney slices in Ringer-phosphate solution containing 0.02 M malonate. The inhibitors were left in contact with the tissue for 20 minutes previous to addition of α-ketoglutarate. α-Ketoglutarate, 0.01 M. The figures are given in c.mm. per mg. of dry tissue in 2 hours.

Inhibitor	O_2 uptake		Inhibition	α-Ketoglutarate utilization		Inhibition
	Control	Inhibitor		Control	Inhibitor	
	c mm.	*c mm*	*per cent*	*c.mm.*	*c mm.*	*per cent*
Arsenical V, 1×10^{-4} M	13.6	3.8	72	78.2	1.3	98
p-Chloromercuribenzoate, 2.5×10^{-4} M	14.7	4.4	70			
Iodoacetamide, 2.0×10^{-3} M	14.7	0.6	96			

Alcohol Oxidase—In 1935 Wagner-Jauregg and Möller (9) found that $CuSO_4$ inhibited alcohol oxidase, the inhibition being reversed by glutathione. This observation added to that of Dixon (10) of inhibition by iodoacetate indicates that the enzyme is an —SH protein. Further evidence in support of this contention is given in Fig. 2. The activating protein of alcohol oxidase, prepared according to Negelein and Wulff (33), was inhibited completely by 5×10^{-4} M *p*-carboxyphenylarsine oxide and partially by 2.5 × 10^{-4} M. In contrast to this powerful inhibition, 5 × 10^{-4} M arsenite had very little effect on the rate of reduction of diphosphopyridine nucleotide.

Lutwak-Mann (34) made the interesting discovery that while yeast alcohol dehydrogenase was readily inhibited by iodoacetate, liver alcohol dehydrogenase was not affected by this reagent. Lutwak-Mann's findings were confirmed by studying the rate of reduction of diphosphopyridine nucleotide by alcohol, activated by liver alcohol dehydrogenase (in the presence of 1 × 10^{-3} M semicarbazide to bind the acetaldehyde formed during the oxidation of alcohol). The rate was unaffected by the addition of organic arsenicals, *p*-chloromercuribenzoate, and iodoacetamide. The inhibition of yeast alcohol oxidase by —SH reagents and the ineffectiveness on liver alcohol oxidase show that, while the protein of the alcohol oxidase from yeast required —SH groups for enzyme activity, the protein of the same enzyme from liver needs no —SH groups.

TABLE VII

Inhibition of Malate Oxidation by —SH-Combining Substances

Enzyme from pigeon heart, 3.0 cc.; malate, 1 M, 0.3 cc.; semicarbazide, 0.1 M, 0.5 cc.; diphosphopyridine nucleotide (0.2 per cent), 0.5 cc.; 0.1 M phosphate, pH 7.4, enough to make 4.95 cc.; gas phase, air; 38°. The blank gave no O_2 uptake.

Inhibitor	Concentration	O_2 uptake		Inhibition
		Control	Inhibitor	
	M	*c mm*	*c mm*	*per cent*
Arsenical I	5 × 10^{-4}	105.2	32.1	69.5
" V	2 × 10^{-4}	85.2	41.8	51.0
p-Chloromercuribenzoate	2 × 10^{-4}	85.2	0	Complete
Iodoacetamide	3 × 10^{-3}	109.0	109.4	None

Effect of —SH Reagents on Other Protein Components of Oxidation Enzymes —It is known that most of the oxidation enzymes which take part in carbohydrate metabolism are complex systems made up of an activating protein (dehydrogenase) with which a prosthetic group might be combined, and oxidation-reduction systems (flavins, iron porphyrins) united to proteins. Thiol groups essential for enzyme activity, reported previously and those reported in this paper, have been found exclusively in activating proteins. The activity of cytochrome oxidase, as determined by the rate of oxidation of reduced cytochrome *c* was completely unaffected by *p*-chloromercuribenzoate, arsenicals, and iodoacetamide. The activity of heart flavoprotein (flavin dinucleotide) as tested with the activating protein of lactate oxidase, diphosphopyridine nucleotide, and methylene blue was also found unaffected on addition of the same reagents. The activity of catalase was also unaffected by the addition of —SH reagents.

Furthermore, enzymes of less complex composition (metalloproteins) were also found to require no —SH groups for enzyme activity. Among these enzymes, uricase, polyphenol oxidase, carbonic anhydrase, and phosphatases were not affected by the addition of —SH reagents.

Lactic and isocitric dehydrogenases were also found not to be affected in their activity on addition of —SH reagents. The first enzyme was prepared from heart, the rate of diphosphopyridine nucleotide reduction being measured. The second enzyme was prepared from liver; here the rate of methylene blue reduction was the measure of enzyme activity with citrate as substrate.

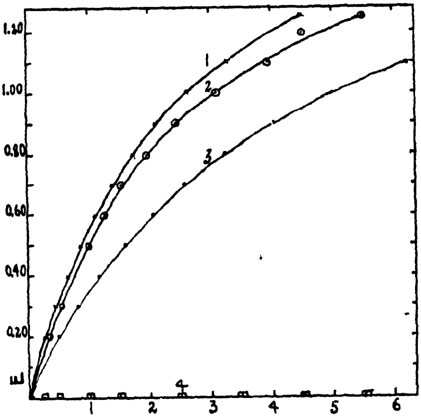


FIG. 2. The inhibition of yeast alcohol oxidase (activating protein) by organic arsenicals 0.1 cc. of activating protein (20 γ of enzyme of 28 per cent purity); 2 mg of diphosphopyridine nucleotide of 40 per cent purity; 0 003 M semicarbazide; pyrophosphate buffer, 0.03 M, pH 7.9; total volume, 4 cc. *E*, extinction coefficient at 3450 A (ordinate); abscissa, time in minutes Curve 1, control; Curve 2, 5 × 10^{-4} M sodium arsenite; Curve 3, 2.5 × 10^{-4} M *p*-carboxyphenylarsine oxide; Curve 4, 5 × 10^{-4} M *p*-carboxyphenylarsine oxide.

Reactivation of —SH Enzymes—When the —SH groups of the —SH enzymes are rendered inactive either by oxidation or mercaptide formation, they may under certain conditions be regenerated with complete reactivation of enzyme activity by the addition of reducing agents (ascorbic acid, H_2S, cyanide) or mercaptide-forming substances (thiols). In this last case the degree of reactivation is determined, among other factors, by the affinity of the enzyme and of the thiol to the mercaptide-forming inhibitor (dissociation constants of the respective mercaptides). Inhibitor and reactivator compete for the —SH groups. If the competitor has greater affinity for the inhibitor than the enzyme, or is used in large concentrations, reactivation of the enzyme will result.

Reactivation of the —SH enzymes described in this paper was attempted with either glutathione or cysteine in concentrations about 10 times that of the —SH inhibitor. As a rule the thiol compounds were added 10 minutes after addition of the inhibitor, time sufficient to produce maximum inhibition of the enzyme system. In every case blanks were run with enzyme plus thiol. Succinoxidase, inhibited by organic arsenicals or quinone, was completely reactivated on addition of glutathione (50 times the concentration of the inhibitor) (Table VIII). This reactivation produced by glutathione was instantaneous and in no way connected with its oxidation by metal catalysts in the enzyme, since the enzyme suspension showed no appreciable O_2 uptake on addition of glutathione without succinate during the course of the experiments (20 minutes).

Enzymes for Pyruvate Metabolism—Before the —SH nature of pyruvate oxidase was demonstrated, it was found that pyruvate oxidation and pyruvate condensation by washed pigeon liver were considerably increased on addition of glutathione (35). Undoubtedly, during the process of grinding and washing, the —SH groups of the enzyme were partially oxidized either by atmospheric oxygen or heavy metal impurities. The oxidation of pyruvate by gonococci, 94 per cent inhibited by Arsenical IV (1×10^{-4} M), was reactivated 15 per cent on addition of glutathione (Table VIII). When the arsenical concentration was diminished so as to produce only partial inhibition (40 per cent), the reactivation by glutathione increased to 77 per cent. The oxidation of pyruvate by ground washed chicken liver, inhibited by *p*-chloromercuribenzoate, was partially reactivated by glutathione (39 per cent reactivation). The oxidation of pyruvate by rat liver slices, inhibited by Arsenical III (75 per cent inhibition), was reactivated by glutathione (97 per cent), with a concentration 10 times that of the arsenical. The inhibition of yeast carboxylase by Arsenical III was abolished on addition of cysteine and H_2S (8 times the inhibitor concentration); the inhibition produced by *p*-chloromercuribenzoate was abolished on addition of glutathione. Similar reactivation of the —SH enzymes for pyruvate condensation (acetoacetate synthesis, acetylmethylcarbinol formation) was observed on addition of glutathione, about 10 times the concentration of the inhibitor. Pyruvate dismutation was only partially reactivated even when the inhibitor concentration was lowered so as to give only 35 per cent inhibition. There were technical difficulties when reactivation of α-ketoglutarate formation was attempted, because the determination of α-ketoglutarate (oxidized to succinate) was performed with the aid of succinoxidase. When the succinate was extracted with ether, there appeared to be also extraction of the sulfhydryl inhibitor.

TABLE VIII

Reactivation of —SH Enzymes Inhibited by Mercaptide-Forming Substances

The conditions of the experiments were similar to those described in the preceding tables.

Enzyme	Inhibitor	Inhibition	Reactivator	Reactivation
		per cent		*per cent*
Succinoxidase	Arsenical I, 5×10^{-5} M	80	Glutathione, 1×10^{-3} M	Complete
	“ “ 5×10^{-5} “	80	Cysteine, 1×10^{-2} M	25
	Benzoquinone, 5×10^{-5} M	80	Glutathione, 2×10^{-3} M	Complete
Pyruvate oxidase (gonococci)	Arsenical IV, 1×10^{-4} M	94	“ 1×10^{-3} “	15
	“ I, 1.5×10^{-5} “	40	“ 1×10^{-3} “	77.5
“ “ (ground, washed chicken liver)	*p*-Chloromercuribenzoate, 5×10^{-4} M	92	“ 2.5×10^{-3} M	39
Pyruvate oxidase (liver slices)	Arsenical III, 5×10^{-4} M	75	“ 5×10^{-3} M	97
Yeast carboxylase	“ “ 1.2×10^{-3} M	89 5	Cysteine, 1×10^{-2} M	72
	“ “ 1.2×10^{-3} “	89.5	H_2S, 1×10^{-2} M	97
	p-Chloromercuribenzoate, 1×10^{-3} M	95	Glutathione, 1×10^{-2} M	92
Pyruvate condensation (acetylmethylcarbinol formation)	“ 1×10^{-3} “	85	“ 1×10^{-2} “	Complete
	Arsenical IV, 1×10^{-3} M	46	“ 1×10^{-2} “	“
Pyruvate condensation (acetoacetate synthesis)	“ III, 1×10^{-3} “	Complete	“ 5×10^{-3} “	“
	p-Chloromercuribenzoate, 1×10^{-3} M	“	“ 1×10^{-2} “	36
Pyruvate dismutation	Arsenical III, 5×10^{-4} M	35	“ 5×10^{-3} “	54
Malate oxidase	“ I, 5×10^{-4} M	69	“ 5×10^{-3} “	71
Adenosinetriphosphatase (myosin)	*p*-Chloromercuribenzoate, 1×10^{-3} M	Complete	“ 1×10^{-2} “	Complete

Malate oxidase inhibited by Arsenical I was reactivated by glutathione at a concentration 10 times that of the inhibitor. It has been shown that adenosinetriphosphatase (myosin) is an —SH enzyme (36). This enzyme inhibited by *p*-chloromercuribenzoate was completely reactivated on addition of glutathione (Table VIII).

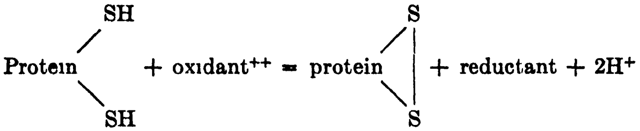
The —SH groups are essential not only for the enzymatic activity, but also for the stability of the protein. When the sulfhydryl groups have been destroyed by oxidation or mercaptide formation, complete reactivation of the enzyme is possible only within certain time limits, beyond which the protein loses enzyme activity irreversibly. This must be the reason for the partial reactivations. Succinoxidase, for example, completely inhibited by *p*-chloromercuribenzoate, was completely reactivated by glutathione when the latter was added 10 to 15 minutes after the mercurial; 85 per cent reactivated when added 30 minutes after the inhibitor; 70 per cent reactivated when added 60 minutes later.

DISCUSSION

It is known that the —SH groups of amino acids possess a certain resistance to the action of mild oxidizing agents, possibly because of the formation upon oxidation of the —S—S— bond. In the protein molecule this resistance may be increased by a variety of factors: the position of the —SH groups with respect to each other, a physical factor which affects not only the rate of oxidation but also the possibility of oxidation; the electronegativity values of the groups neighboring the —SH groups; steric hindrances of various sorts. In some instances the —SH groups are so situated that these factors contribute to the loosening of the —SH bond, ready oxidation being the consequence. In other cases these factors add up to the tightening of the bond and make oxidation impossible unless by rupture of some H bonds in the protein molecule (denaturation) the hindering factors have been abolished. For convenience we may then divide the protein —SH groups into two types, *freely reactive —SH groups*, which are oxidized and give the nitroprusside test when the protein is in the native state, and *sluggish —SH groups*, which are not oxidized and do not give the nitroprusside test except after denaturation of the protein.

In general, the oxidizing agents usually employed for the titration of these —SH groups (ferricyanide, porphyrindine) react only with those present in the native protein; sometimes even some of these —SH groups fail to react. The alkylating agents react with the —SH groups as second order reactions (Hellström (37)), and large concentrations are required for complete reaction; they react also with —NH_2 groups, especially iodoacetate. The mercaptide-forming compounds seem to possess a greater affinity for —SH groups, as they attack those that have been left untouched by the oxidizing agents. The affinity of these sulfhydryl groups for the —SH reagents varies from protein to protein, depending among other factors on their position in the molecule and their degree of dissociation. In some instances only certain —SH groups are essential for enzyme activity, as Hellerman found in urease. It is essential to have these facts in mind when detecting —SH enzymes; otherwise erroneous conclusions may be reached. Reagents must be selected that do not produce protein denaturation and that react with all types of —SH groups; in the case of mercaptide formation reactivations must be produced. It is only when various selected reagents have produced enzyme inhibition that satisfactory conclusions can be reached.

The oxidizing agents so widely used for the titration of —SH groups in proteins have proved to be less effective in detecting —SH enzymes. They oxidize the —SH groups to the disulfide,



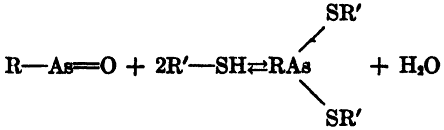
$$\text{Protein}\begin{matrix}\diagup\text{SH}\\ \diagdown\text{SH}\end{matrix} + \text{oxidant}^{++} = \text{protein}\begin{matrix}\diagup\text{S}\\ \diagdown\text{S}\end{matrix}\!\Big| + \text{reductant} + 2\text{H}^{+}$$

thus requiring the presence of —SH groups close enough for bond formation. There may be native protein molecules in which, due to the particular stereochemical structure, the individual —SH groups are not sufficiently close together to form an S—S bond. In these cases oxidizing agents might be ineffective in detecting —SH groups. For example, ferricyanide, used by Anson (38) and by Mirsky (39) for the titration of —SH groups in proteins, has no effect on the enzymatic activity of urease (Hellerman (5)) and of choline oxidase, enzymes requiring the presence of —SH groups for activity, and has weak inhibitory action on succinoxidase. Porphyrindine, the reagent used by Greenstein (40), has no effect on the activity of papain, an —SH enzyme (41).

Iodoacetate has been considered a valuable and almost specific reagent for the detection of —SH enzymes. When acting on proteins it is assumed that it reacts by substitution of the H of —SH by the carboxymethyl group, protein—SH + CH_2ICOOH = protein—SCH_2COOH + HI (Rapkine (42), Dickens (43)). Under certain conditions this alkylation of —SH groups is more rapid than that of amino groups but reaction with —SH groups does not preclude reaction with —NH_2 groups, as has been shown in experiments in which *p*-chloromercuribenzoate and iodoacetate were added together to succinoxidase. Moreover, Smythe (44) has shown the great variation in the rate of interaction of iodoacetate with even simple alkyl mercaptans. Even iodoacetamide (a better —SH reagent in this group) is a poor reagent for the detection of —SH groups because of the irregularity of its action.

(It does not react with the —SH groups of egg albumin at pH 7.3 (Rosner (45)), while it abolishes 40 per cent of them at pH 9 (Anson (46).) Similar variations are found when iodoacetamide is used for detecting —SH enzymes. At a concentration 3 times higher than that required for abolition of the active —SH groups of urease, iodoacetamide had no effect on the enzyme activity (Hellerman *et al.* (1)). It had no effect on the enzyme activity of malate oxidase or of adenosinetriphosphatase, both enzymes requiring essential —SH groups.

The third group of reagents consists of those which form addition compounds. The trivalent organic arsenicals used in the experiments reported here belong to this group. They combine reversibly with —SH groups (Voegtlin, Dyer, and Leonard (47), Cohen, King, and Strangeways (48)),



$$R{-}As{=}O + 2R'{-}SH \rightleftarrows RAs\begin{matrix} \diagup SR' \\ \diagdown SR' \end{matrix} + H_2O$$

giving compounds which readily dissociate on increase of the pH value of the solution, or when the pH is kept constant, on addition of another thiol compound possessing greater affinity for the arsenical. They do not react with the —SH groups of native egg albumin but combine with them on denaturation (Rosenthal (49)). They were used first as enzyme inhibitors by Bersin (50). The organic arsenicals are powerful tools in the quest for —SH groups in enzymes not only because they form reversible thioarsenites but also because their power to link with —SH groups approaches that of Hellerman's *p*-chloromercuribenzoate

From the effect of the inhibitors on the enzyme systems reported here, a rough classification could be made of the —SH reagents: (1) mild oxidizing agents (ferricyanide, porphyrindine, 2,6-dichlorophenol indophenol) which act only on those —SH groups close enough to allow S—S formation; (2) certain alkylating reagents (iodoacetamide), which, though combining with more —SH groups than the former, leave some unattacked; (3) reagents like arsenicals and *p*-chloromercuribenzoate which combine with all the available —SH groups in the native protein. Of all the enzyme systems studied, pyruvate oxidase and the enzyme for pyruvate condensation (acetoacetate formation) were the most sensitive to the action of enzyme inhibitors; in fact, all three classes of reagents produced enzyme inhibition. Succinoxidase and most of the other —SH enzymes are not significantly inhibited by low concentrations of ferricyanide; they are inhibited by the second and third class of —SH reagents. Finally urease is not inhibited by mild oxidizing agents or arsenicals; the active sulfhydryl groups are abolished only by the powerful —SH reagent, *p*-chloromercuribenzoate. These examples show that a step by step abolition of the —SH groups of proteins can be accomplished by the use of reagents possessing different affinities for these groups.

The experiments presented here, as well as those found in the literature, demonstrate that the presence of —SH groups is essential for the activity of a large number of enzymes concerned with the metabolism of carbohydrate. In the first phase of fermentation (glycogen to pyruvate) the following enzymes are —SH enzymes: muscle phosphorylase, phosphoglucomutase, hexokinase, phosphoglyceraldehyde oxidase. In the second phase of fermentation (pyruvate to lactate or pyruvate to acetaldehyde + CO_2), the enzyme leading to alcohol fermentation, carboxylase, is an —SH enzyme, while the enzyme leading to lactate formation is not. In the oxidative phase of carbohydrate metabolism there is some evidence for considering hexose monophosphate oxidase an —SH enzyme, the evidence being inhibition by $CuCl_2$ and iodoacetate (reported by von Euler and Adler). Evidence has been presented here that pyruvate oxidation, pyruvate condensation, malate oxidation, and α-ketoglutarate oxidation require —SH enzymes. Succinate oxidation and yeast alcohol oxidation also require —SH enzymes. Among the phosphorylases which take part in the transphosphorylations necessary for the continuous fermentative and oxidative processes, the following are —SH enzymes: adenosinetriphosphatase (myosin) and myokinase. In short, so many steps in the the series of reactions occurring during carbohydrate metabolism require —SH enzymes, that it may be concluded that sulfhydryl enzymes occupy an important place in the breakdown and synthesis of glycogen.

SUMMARY

Reagents used for the detection of essential —SH groups in enzymes have been classified according to their affinity for these groups. Among the enzymes taking part in carbohydrate metabolism the following were inhibited by the —SH reagents: pyruvate oxidase, carboxylase, α-ketoglutarate oxidase, malate oxidase, adenosinetriphosphatase (myosin), and the enzymes for pyruvate condensation. Enzymes not inhibited by the —SH reagents were lactate oxidase, isocitrate oxidase, acid phosphatase, carbonic anhydrase, polyphenol oxidase, and catalase. Essential —SH groups were found only in the activating proteins (the proteins which combine with the substrate). Cytochrome oxidase and flavoproteins were not inhibited by —SH reagents.

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BIBLIOGRAPHY

1. Hellerman, L., Chinard, F. P., and Deitz, V. R., *J. Biol. Chem.*, **147**, 443 (1943).
2. Hopkins, F. G., Morgan, E. J., and Lutwak-Mann, C., *Biochem. J.*, **32**, 1829 (1938).
3. Rapkine, L., *Biochem. J.*, **32**, 1729 (1938).
4. Bersin, T., in Nord, F. F., and Weidenhagen, R., Ergebnisse der Enzymforschung, Leipzig, **4**, 68 (1935).
5. Hellerman, L., *Physiol. Rev.*, **17**, 454 (1937); Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, **7**, 165 (1939).
6. Hopkins, F. G., and Morgan, E. J., *Biochem. J.*, **32**, 611 (1938).
7. Lehmann, H., *Biochem. J.*, **33**, 1241 (1939).
8. von Euler, H., and Adler, E., *Z. physiol. Chem.*, **232**, 10 (1935).
9. Wagner-Jauregg, T., and Möller, E. F., *Z. physiol. Chem.*, **236**, 222 (1935).
10. Dixon, M., *Nature*, **140**, 806 (1940).
11. Barron, E. S. G., *Bol. Soc. quím. Peru*, **6**, 7 (1940).
12. Anson, M. L., *Science*, **90**, 142 (1939).
13. Labes, R., and Freisburger, H., *Arch. exp. Path. u. Pharmakol.*, **156**, 226 (1930).
14. Morgan, E. J., and Friedmann, E., *Biochem. J.*, **32**, 733 (1938).
15. Hellerman, L., Chinard, F. P., and Ramsdell, P. A., *J. Am. Chem. Soc.*, **63**, 2551 (1941).
16. Lu, G. D., *Biochem. J.*, **33**, 249 (1939).
17. Berenblum, I., and Chain, E., *Biochem. J.*, **32**, 295 (1938).
18. Krebs, H. A., *Biochem. J.*, **32**, 108 (1938).
19. Edson, N. L., *Biochem. J.*, **29**, 2082 (1925).
20. Quastel, J. H., and Wheatley, A. H. M., *Biochem. J.*, **32**, 936 (1938).
21. Michaelis, L., and Schubert, M. P., *J. Biol. Chem.*, **106**, 331 (1934).
22. Potter, V. R., and DuBois, K. P., *J. Gen. Physiol.*, **26**, 391 (1943).
23. Barron, E. S. G., *Enzymologia*, **3**, 149 (1943).
24. Peters, R. A., in Needham, J., and Green, D. E., Perspectives of biochemistry, Cambridge, 41 (1937).
25. Barron, E. S. G., *J. Biol. Chem.*, **113**, 695 (1936).
26. Snell, J. M., and Weissberger, A., *J. Am. Chem. Soc.*, **61**, 450 (1939).
27. Barron, E. S. G., Goldinger, J. M., Lipton, M. A., and Lyman, C. M., *J. Biol. Chem.*, **141**, 975 (1941).
28. Green, D. E., Westerfeld, W. W., Vennesland, B., and Knox, W. E., *J. Biol. Chem.*, **145**, 69 (1942).
29. Evans, E. A., Jr., and Slotin, L., *J. Biol. Chem.*, **141**, 439 (1941).
30. Greenberg, L. D., and Rinehart, J. F., *Proc. Soc. Exp. Biol. and Med.*, **43**, 495 (1940).
31. Krebs, H. A., and Johnson, W. A., *Enzymologia*, **4**, 148 (1937).
32. Green, D. E., *Biochem. J.*, **30**, 2095 (1936).
33. Negelein, E., and Wulff, H. J., *Biochem. Z.*, **293**, 351 (1937).
34. Lutwak-Mann, C., *Biochem. J.*, **32**, 1364 (1938).
35. Barron, E. S. G., and Singer, T. P., *Science*, **97**, 356 (1943).
36. Singer, T. P., and Barron, E. S. G., *Proc. Soc. Exp. Biol. and Med.*, **56**, 120 (1944).
37. Hellström, N., *Z. phys. Chem.*, *Abt. A*, **157**, 242 (1931).
38. Anson, M. L., *J. Gen. Physiol.*, **24**, 399 (1940–41).
39. Mirsky, A. E., *J. Gen. Physiol.*, **24**, 709 (1940–41).
40. Greenstein, J. P., *J. Biol. Chem.*, **125**, 501 (1938).
41. Balls, A. K., and Lineweaver, H., *J. Biol. Chem.*, **130**, 66
42. Rapkine, L., *Compt. rend. Soc. biol.*, **112**, 1294 (1933).

43. Dickens, F., *Biochem. J.*, **27**, 141 (1933).
44. Smythe, C. V., *J. Biol. Chem.*, **114**, 601 (1936).
45. Rosner, L., *J. Biol. Chem.*, **132**, 657 (1940).
46. Anson, M. L., *J. Gen. Physiol.*, **23**, 321 (1940).
47. Voegtlin, C., Dyer, H. A., and Leonard, C. S., *Pub. Health Rep., U. S. P. H. S.*, **38**, 1911 (1923).
48. Cohen, A., King, H., and Strangeways, W. I., *J. Chem. Soc.*, 3043 (1931).
49. Rosenthal, S. M., *Pub. Health Rep., U. S. P. H. S.*, **47**, 241 (1932).
50. Bersin, T., *Z. physiol. Chem.*, **222**, 177 (1933).

STUDIES ON BIOLOGICAL OXIDATIONS

XX. SULFHYDRYL ENZYMES IN FAT AND PROTEIN METABOLISM

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The large number of enzyme systems concerned with carbohydrate metabolism which require the presence of —SH groups in the molecule of the activating protein (dehydrogenase) made it imperative to determine whether the presence of these same groups was required in the case of enzymes concerned with the metabolism of fats and proteins. Very little is known regarding the necessity of —SH groups for the activity of fat oxidation enzymes. Green and coworkers (1) reported that β-hydroxybutyric dehydrogenase is inhibited by 0.03 M iodoacetate and arsenite, both —SH reagents of doubtful value. According to Yosii (2), an enzyme in adipose tissue responsible for the oxidation of higher fatty acids is inhibited by heavy metals (Ag, Cu, Hg), an indication that the enzyme might require —SH groups. The water-soluble enzyme extracted from liver by Konrad-Lang (3), which oxidizes stearate and palmitate, was not inhibited by iodoacetate. Much work has been done, however, on enzymes concerned with protein metabolism. Suffice it to recall that Perlzweig's discovery (4) of —SH groups in urease initiated the extensive experimental work on the detection of essential —SH groups in hydrolytic and proteolytic enzymes. Very little information is available on the necessity of —SH groups in other enzymes concerned with protein metabolism, the oxidation of amino acids, and transamination. Friedenwald and Herrmann (5) have demonstrated that amine oxidase is a sulfhydryl enzyme. We had reached the same conclusion in experiments performed previously to Friedenwald's report.

In this paper we present the results of a systematic investigation of the necessity of —SH groups for the activity of some enzymes concerned with the metabolism of fats and proteins. From these studies, and from those previously reported, it may be concluded that the presence of —SH groups in the protein component of a large number of enzymes is essential for activity.

The great lability of many of these —SH groups (as shown by the difficulty of preparing many of the —SH enzymes unless a reducing substance is present), the restoration of enzyme activity on addition of glutathione, and the almost universal presence of this tripeptide as an intracellular constituent make plausible the suggestion that one of the functions of glutathione is that of maintaining the great number of the —SH enzymes at optimum activity. Glutathione would then be necessary for the metabolism of all foodstuffs (carbohydrates, fats, proteins), since many of the reactions of the metabolic cycle are catalyzed by —SH enzymes.

Sulfhydryl Enzymes in Fat Metabolism

There is, it seems, no field of enzymology more lacking in information than that of enzymes concerned with the metabolism of fats. Enzymes from liver or from bacteria oxidize saturated, high molecular weight fatty acids to the corresponding unsaturated fatty acids; some enzymes from plants and bacteria readily oxidize certain unsaturated fatty acids; and a number of bacteria readily oxidize saturated lower fatty acids. However, the components of the enzyme systems as well as the mechanisms of enzyme action are still unknown.

Stearate Oxidase—The enzyme for the *oxidation of stearate* was tested in the aqueous extract obtained according to the method of Konrad-Lang (3), and in suspensions of washed *Bacillus coli.*

The liver extract of Konrad-Lang produces activation of stearate in the presence of added adenosine triphosphate; a reversible dye must be used as the oxidizing agent. The time of methylene blue reduction was observed in Thunberg tubes kept in a vacuum at 38°. Complete reduction occurred in the control tubes in 11 minutes. In the presence of 1×10^{-4} M *p*-chloromercuribenzoate the activity of the enzyme was completely lost; with 1×10^{-3} M iodoacetate the reduction time was 14 minutes; with 1×10^{-3} M *p*-hydroxyphenylarsine oxide 75 per cent reduction occurred in 70 minutes. Since the dye reduction proceeds as a first order reaction, the degree of inhibition was calculated from the reduction time (Table I).

Washed suspensions of *Bacillus coli* oxidize stearate and oleate (6). The oxidation of stearate was completely inhibited by 5×10^{-5} M *p*-chloromercuribenzoate; 1×10^{-3} M *p*-carboxyphenylarsine oxide produced an inhibition of 77 per cent; 1×10^{-3} M iodoacetate inhibited by 47 per cent; and 2×10^{-3} M iodoacetamide gave an inhibition of 80 per cent. The oxidation of oleate was also inhibited by all these reagents to about the same extent as the inhibition of stearate; alloxan (1×10^{-2} M) inhibited by 33 per cent (Table II).

The oxidation of oleic acid by an enzyme extracted from peanuts was not affected by any of the —SH reagents tested.

Acetate Oxidation—The oxidation of acetic acid by yeast seems to start, according to Lynen (7), by a condensation of acetate with oxalacetate and formation of isocitric acid. The oxidation of isocitric acid would give α-ketoglutaric acid which in turn would be oxidized to succinic acid. Acetate would thus enter the C_4-dicarboxylic acids series of catalysts. The oxidation of acetate by washed bakers' yeast was completely inhibited by *p*-chloromercuribenzoate (1.5×10^{-3} M) and by *p*-carboxyphenylarsine oxide (2×10^{-3} M). Since the oxidation of isocitric acid is not inhibited by these substances, it may be concluded that the condensation reaction

TABLE I

Effect of —SH Reagents on Stearate Dehydrogenase from Rat Liver

Enzyme, 1 cc. of dialyzed rat liver extract + 1 cc. of 10^{-2} M sodium stearate + 0.25 cc. of phosphate buffer, 0.1 M, pH 7.4, containing 50 γ of adenosine triphosphate. Methylene blue, 0.15 cc. of 1:5000 solution.

Inhibitor	Concentration of inhibitor	Reduction		Inhibition
	M	*per cent*	*min.*	*per cent*
Blank			∞	
None		100	11	
p-Chloromercuribenzoate	1×10^{-4}	100	∞	Complete
Iodoacetate	1×10^{-3}	100	14	11
p-Carboxyphenylarsine oxide	1×10^{-3}	75	70	78

TABLE II

Inhibition of Fatty Acid Oxidation by Bacillus coli communis

2.2 cc. of twice washed bacteria suspended in 0.04 M Ringer-phosphate solution, pH 7.4; substrate, 1 cc. of 1×10^{-2} M sodium salt; buffer, 0.05 M phosphate, pH 7.4, to 4 cc.; 38°; gas phase, air; duration of experiments, 40 minutes.

Inhibitor	Concentration of inhibitor	Substrate	O_2 consumption (blank subtracted)	Inhibition
	M		*c.mm.*	*per cent*
None		Stearate	46.8	
p-Carboxyphenylarsine oxide	1×10^{-3}	"	10.6	77.4
None		Oleate	57.4	
p-Carboxyphenylarsine oxide	1×10^{-3}	"	15.6	72.9
None		Stearate	55.0	
p-Chloromercuribenzoate	5×10^{-5}	"	0	100
None		Oleate	70.8	
p-Chloromercuribenzoate	5×10^{-5}	"	0	100
None		Stearate	59.5	
Iodoacetate	1×10^{-3}	"	31.4	47.3
Iodoacetamide	2×10^{-3}	"	11.8	80.2
None		Oleate	85.5	
Iodoacetate	1×10^{-3}	"	49.2	42.5
Iodoacetamide	2×10^{-3}	"	15.8	81.5
None		"	72.9	
Alloxan	1×10^{-2}	"	48.5	33.5

of acetate with oxalacetate, first phase of acetate oxidation, requires an —SH enzyme.

Corynebacterium creatinovorans strain NC, the soil bacteria discovered by Dubos and Miller (8), increases its ability to oxidize acetate when grown in a medium containing acetate as the sole source of carbon. The oxidation of acetate by these bacteria was completely inhibited by *p*-chloromercuribenzoate (5×10^{-4} M), 94 per cent by iodoacetamide (5×10^{-3} M), and 98 per cent by 3-amino-4-hydroxyphenyldichloroarsine hydrochloride

TABLE III

Effect of —SH Reagents on Acetate Oxidation by Bakers' Yeast, and by Corynebacterium creatinovorans

0.3 cc. of washed bakers' yeast (7.2 mg. dry weight) suspended in water; 1.0 cc. of 0.2 M PO_4, pH 6.8; 0.3 cc. of 0.1 M sodium acetate; inhibitors and water to 3.0 cc.; 38°; gas phase, air; duration of experiments, 60 minutes. The —SH reagents were left in contact with the yeast for 10 minutes before the glutathione was added. Appropriate blanks were subtracted. The *Corynebacterium creatinovorans* was grown on acetate broth. 1 cc. of washed bacteria was used per vessel. Buffer, 0.02 M phosphate, pH 7.4; substrate, 0.01 M acetate; 38°; duration of experiments, 30 minutes.

Inhibitor and reactivator	Oxygen consumption		Inhibition
	Control	Inhibitor	
	c mm.	*c mm.*	*per cent*
1. Bakers' yeast			
p-Chloromercuribenzoate, 1.5×10^{-3} M	207	0	Complete
" 1×10^{-4} M	203.8	73.4	64
p-Carboxyphenylarsine oxide, 2×10^{-3} M	207	5.9	97
" " 5×10^{-4} "	203.8	160.3	21
2. *Corynebacterium creatinovorans*			
p-Chloromercuribenzoate, 5×10^{-4} M	186.0	0	Complete
3-Amino-4-hydroxyphenyldichloroarsine hydrochloride, 2×10^{-4} M	195.4	4.4	98.5
Iodoacetamide, 5×10^{-3} M	186.0	11.8	93.7

(Table III). If oxidation of acetate by these bacteria follows the same path as in yeast, these experiments would be confirmatory of those with yeast.

β-Hydroxybutyrate Dehydrogenase—It was shown by Green that β-hydroxybutyrate dehydrogenase is inhibited by iodoacetate and arsenite, an indication that the enzyme requires —SH groups for activity. In agreement with this assumption we found a complete inhibition of this oxidation by *p*-chloromercuribenzoate (5×10^{-4} M) and *p*-carboxyphenylarsine oxide (5×10^{-4} M), and a large inhibition produced by iodoacetamide (72 per cent with 5×10^{-3} M iodoacetamide) (Table IV). In these experiments the oxidation of β-hydroxybutyrate was measured by the O_2 uptake in the presence of diphosphopyridine nucleotide. The preparation contained the flavin dinucleotide and cytochrome system necessary for electron transfer to molecular oxygen.

Pancreatic Lipase—In an extensive study of various enzyme inhibitors on the hydrolysis of tripropionin by pancreatic lipase, Weinstein and Wynne (9) found that heavy metals (Cu^{++}, Fe^{+++}, Hg^{++}), and the acid halides, chloro-, bromo-, and iodoacetic acids, inhibited the enzyme; on the other hand KCN, $Na_2S_2O_4$, cysteine, and thioglycolic acid activated it. Since acid halides combine with both —SH and —NH_2 groups at H^+ ion concentrations at which the experiments were conducted (10), these authors left unanswered the mechanism of inhibition. The experiments reported in Table V were performed at pH 7.4. *p*-Chloromercuribenzoate (1×10^{-3} M) inhibited the enzyme by 38 per cent; *p*-aminophenyldichloroarsine hydrochloride (1×10^{-3} M) inhibited 62 per cent; and 3-amino-4-hydroxyphenyldichloroarsine hydrochloride (1×10^{-3} M) produced an inhibition of 52 per cent. Iodoacetamide (5×10^{-3} M) had no effect at all.

TABLE IV

Inhibition of β-Hydroxybutyrate Oxidase by —SH Reagents

Enzyme, prepared according to Green (1), 3 cc.; substrate, 3.3×10^{-3} M sodium β-hydroxybutyrate; buffer, pyrophosphate, pH 7.4; 0.3 cc. of 0 5 per cent diphosphopyridine nucleotide; 38°; duration of experiments, 25 minutes.

Inhibitor	Concentration of inhibitor	O_2 uptake	Inhibition
	M	*c mm.*	*per cent*
None		76.3	
p-Chloromercuribenzoate	5×10^{-4}	0	Complete
p-Carboxyphenylarsine oxide	5×10^{-4}	0	"
None		66.0	
Iodoacetamide	5×10^{-3}	18.6	72

Sulfhydryl Enzymes in Protein Metabolism

Hellerman (11) and Bersin (12) have dealt extensively with the —SH enzymes of hydrolytic and proteolytic types belonging to this group. Almost no work has been done with the enzymes concerned with oxidation and transamination reactions.

d-Amino acid oxidase as prepared according to Krebs (13) oxidizes alanine quantitatively to pyruvic acid, because the H_2O_2 formed during the reaction is destroyed as soon as formed by the catalase present in the kidney extract. The enzyme purified by Warburg and Christian (14) contains no

TABLE V

Effect of —SH Reagents on Pancreatic Lipase

Experiment I, 2 cc. of enzyme containing 75 mg. of pancreatin; substrate, 4 cc. of cottonseed oil; buffer, 10 cc. of 0.5 M phosphate, pH 7.5; diluted with water to a final volume of 20 cc. After 3 hours shaking at room temperature, alcohol was added to stop the reaction. Titration with NaOH to phenolphthalein. Experiment II, 2 cc. of enzyme containing 100 mg. of pancreatin; substrate, 2 cc. of cottonseed oil; 0.003 M phosphate added to a final volume of 5 cc. Duration of experiment, 20 hours. Experiment III, 2 cc. of enzyme containing 100 mg. of pancreatin, in 0.005 M phosphate, pH 7.5; substrate, 2 cc. of corn oil; final volume, 5 cc.; duration of experiment, 6 hours.

Experiment No.	Inhibitor	Concentration of inhibitor	Fatty acid liberated	Inhibition
		M	*micromoles*	*per cent*
I	None		5220	
	p-Chloromercuribenzoate	1×10^{-3}	3250	38
	Iodoacetamide	5×10^{-3}	5160	0
II	None		2020	
	p-Aminophenyldichloroarsine hydrochloride	1×10^{-3}	760	62
III	None		702	
	3-Amino-4-hydroxyphenyldichloroarsine hydrochloride	1×10^{-3}	339	51.7

TABLE VI

Effect of —SH Reagents on d-Amino Acid Oxidase

Experiment I, 1 cc. of rat kidney extract in 2×10^{-2} M pyrophosphate, pH 8.3, + 0.05 M pyrophosphate to 3 cc.; alanine concentration, 1.7×10^{-1} M (blank values subtracted); time, 30 minutes. Experiment II, at the end of 30 minutes, 0.3 cc. of 3 M acetate buffer, pH 4.81, was added and pyruvate was determined with yeast carboxylase. Experiment III, 2 cc. of enzyme in 0.05 M pyrophosphate, pH 7.96; H_2O to 3 cc.; time, 30 minutes; in all experiments the alanine concentration was 1.7 $\times 10^{-1}$ M; 38°; gas phase, air.

Experiment No.	Inhibitor	Concentration	O_2 uptake or pyruvate utilization	Inhibition
		M	*c.mm.*	*per cent*
I	None		138	
	p-Chloromercuribenzoate (O_2 uptake measured)	1×10^{-4}	30.8	78
II	None		315.1	
	p-Aminophenyldichloroarsine hydrochloride (pyruvate utilization measured)	5×10^{-5}	34.3	90
III	None		127.6	
	Iodoacetamide (O_2 uptake measured)	5×10^{-3}	124	None

catalase and thus the end-product of alanine oxidation is acetic acid, produced by oxidation of pyruvic acid by H_2O_2. Krebs' preparation makes possible the determination of enzyme activity by two simple methods, O_2 consumption and pyruvate analysis. The oxidation of alanine by *d*-amino acid oxidase was largely inhibited by *p*-chloromercuribenzoate (78 per cent by 1×10^{-4} M *p*-chloromercuribenzoate) and by *p*-aminophenyldichloroarsine hydrochloride (90 per cent by the 5×10^{-5} M arsenical). Iodoacetamide, however, had no effect at a concentration of 5×10^{-3} M (Table VI). The O_2 consumption was measured in experiments with the first and third inhibitor; in those with the arsenical, the activity was measured by determining pyruvate formation. This inhibition is due exclusively to mercaptide formation with the —SH groups of the protein moiety. When the enzyme was purified and the flavin dinucleotide was split from the protein according to Negelein and Brömel (15), addition of a large excess of flavin dinucleotide to the protein did not reverse the inhibition of the enzyme. The inhibition disappeared on addition of further amounts of the protein from which the prosthetic group, flavin dinucleotide, had been separated (Fig. 1).

Transaminase—The essential —SH groups in this enzyme were detected with a heart extract prepared according to Cohen (16). Two reactions were studied in these experiments: (1) pyruvate + *l*-glutamate $\rightleftarrows$ α-ketoglutarate + *l*-alanine; (2) oxalacetate + *l*-glutamate $\rightleftarrows$ α-ketoglutarate + *l*-aspartate. In the first reaction, transamination was measured by the determination of pyruvate disappearance (yeast carboxylase); in the second reaction, by determination of the aspartate formation (Cohen's method (17)). The formation of alanine by transamination of pyruvate from *l*-glutamate was inhibited 81 per cent by *p*-carboxyphenylarsine oxide (1×10^{-3} M), and the formation of *l*-aspartate by transamination of oxalacetate from *l*-glutamate was inhibited 49 per cent by *p*-chloromercuribenzoate (1×10^{-3} M). As in the case of *d*-amino acid oxidase, iodoacetamide had no effect on transaminase (Table VII).

Amine Oxidases—Friedenwald and Herrmann (5) reported that tyramine oxidase was inhibited by *p*-chloromercuribenzoate. The experiments reported in Table VIII are in agreement with the conclusions of Friedenwald and Herrmann that amine oxidase is an —SH enzyme. The enzyme used in these experiments was prepared according to Blaschko *et al.* (18). The oxidation of tyramine was inhibited 82 per cent by *p*-chloromercuribenzoate (1×10^{-3} M), 76 per cent by *p*-carboxyphenylarsine oxide, 77 per cent by *p*-aminophenyldichloroarsine hydrochloride (1×10^{-3} M), and 33 per cent by iodoacetamide (5×10^{-3} M). The oxidation of adrenalin was inhibited 44 per cent by 3-amino-4-hydroxyphenyldichloroarsine hydrochloride (5×10^{-4} M).

Diamine oxidase prepared from kidney according to Laskowski (19) was unaffected by *p*-chloromercuribenzoate and by arsenicals (Table VIII).

l-Glutamic Dehydrogenase—The activating protein of *l*-glutamate oxidase is contained in water extracts of pig liver acetone powder prepared according to Dewan (20). When the diphosphopyridine nucleotide is split off, the activity of the enzyme can be measured by following spectrophotometrically the reduction of added diphosphopyridine nucleotide, or by measuring the O_2 consumption with pyocyanine as the oxidizing catalyst. *p*-Chloromercuribenzoate (5×10^{-4} M) and 3-amino-4-hydroxyphenyldichloroarsine hydrochloride (1×10^{-3} M) inhibited completely the activity of the enzyme. Again, iodoacetamide (5×10^{-3} M) had no effect at all (Table IX).

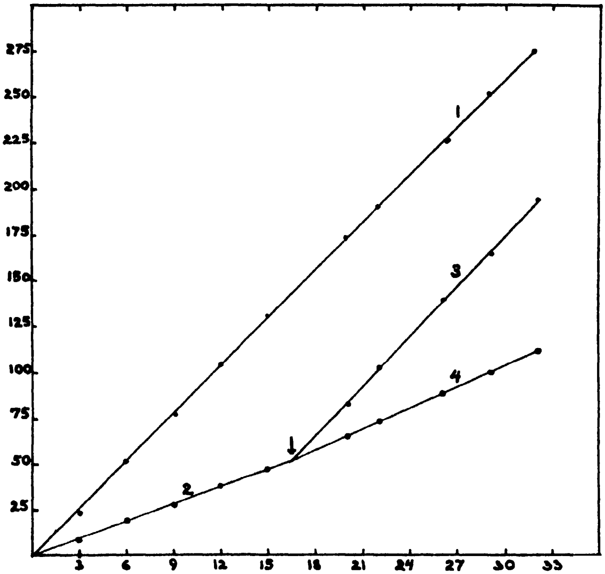

FIG. 1. Combination of the arsenoxide with the protein moiety of *d*-amino acid oxidase. Abscissa, time in minutes; ordinate, c.mm. of O_2 uptake Curve 1, control; Curve 2, 1×10^{-4} M *p*-aminophenyldichloroarsine hydrochloride; Curve 3, same plus 1 cc. of activating protein; Curve 4, 1×10^{-4} M arsenical plus 500 γ of flavin dinucleotide. In the main vessel, 1 cc. of activating protein solution; 500 γ of crude flavin adenine dinucleotide in 0 2 cc ; 0 3 cc. of 0 55 M *dl*-alanine; 1 cc. of 0.05 M pyrophosphate buffer, pH 8 3; 0 5 cc. of arsenical or water In the side arm, 1 cc of activating protein or 500 γ of flavin dinucleotide in 1 cc. The contents of the side arm were tipped in at the end of 16.5 minutes. Temperature 38°; equilibration time, 10 minutes.

Pepsin and trypsin were not inhibited by *p*-chloromercuribenzoate (1×10^{-3} M) or by the arsenicals.

Reactivation of —SH Enzymes with Glutathione—Conclusive evidence of the necessity of —SH groups for enzyme activity is obtained when the

TABLE VII

Effect of —SH Reagents on Transaminase

Experiment I, enzyme in 1 per cent $KHCO_3$, 2 cc.; substrates, 0.5 cc. of 0.12 M pyruvate and 0.2 cc. of 0.3 M *l*-glutamate; time, 90 minutes; 38°. Pyruvate disappearance was determined with yeast carboxylase. Experiments II and III, enzyme, 3 cc.; substrates, 1 cc. of 0.06 M glutamate and 0.3 cc. of 0.2 M oxalacetate; time, 90 minutes; 38°. Aspartate formation was determined with chloramine-T.

Experiment No.	Inhibitor	Concentration	Pyruvate disappearance or aspartate formation	Inhibition
		M	*c.mm.*	*per cent*
I	None		514	
	p-Carboxyphenylarsine oxide	1×10^{-3}	95	81.5
	" "	1×10^{-4}	292	43.2
II	None		1230	
	p-Chloromercuribenzoate	1×10^{-3}	632	48.6
III	None		748	
	Iodoacetamide	5×10^{-3}	747	None

TABLE VIII

Effect of —SH Reagents on Amine Oxidases

Experiment I, enzyme, from guinea pig liver, 2 cc.; buffer, 0.05 M phosphate, pH 7.3; tyramine concentration, 0.036 M, duration of experiments, 100 minutes. Experiment II, enzyme, 3 cc.; buffer, 0.06 M phosphate, pH 7.3; adrenalin concentration, 0.0125 M; duration of experiments, 40 minutes. Experiment III, enzyme, from pig kidneys, in 0.2 M PO_4, pH 7.2; substrate, 0.0066 M histamine; duration of experiments, 270 minutes.

Experiment No	Enzyme-substrate	Inhibitor	Concentration	O_2 uptake	Inhibition
			M	*c mm.*	*per cent*
I	Monoamine oxidase-tyramine	None		188.2	
		p-Chloromercuribenzoate	1×10^{-3}	33.4	82.3
		p-Carboxyphenylarsine oxide	1×10^{-3}	45.2	76.0
		p-Aminophenyldichloroarsine hydrochloride	1×10^{-3}	43.2	77.0
		Iodoacetamide	5×10^{-3}	125.2	33.3
II	Adrenalin	None		119.1	
		3-Amino-4-hydroxyphenyldichloroarsine hydrochloride	5×10^{-4}	66.7	44
III	Diamine oxidase-histidine	None		220	
		p-Carbamylphenylarsine oxide	1×10^{-3}	210	None

enzyme, inhibited by a mercaptide-forming compound, is brought back to full activity on addition of thiols. Reactivation experiments performed with the —SH enzymes discussed in this paper are reported in Table X.

Acetate oxidation by yeast, completely inhibited by *p*-chloromercuribenzoate, was reactivated 75 per cent on addition of glutathione. Acetate oxidation by *Corynebacterium creatinovorans*, inhibited by 3-amino-4-hydroxyphenyldichloroarsine hydrochloride, was reactivated 91 per cent on addition of glutathione (ratio of glutathione to arsenical, 20:1). The activity of β-hydroxybutyrate dehydrogenase, inhibited completely by 5×10^{-4} M *p*-chloromercuribenzoate, was completely restored on addition of 5×10^{-3} M glutathione. In fact, the —SH groups of this enzyme are so labile that in order to obtain preparations of optimal activity it would be necessary to perform the extraction in the presence of glutathione or cysteine. The activity of lipase (commercial pancreatin), inhibited by an organic arsenical, was partially restored (45 per cent reactivation) on addition of glutathione. The activity of *d*-amino acid oxidase, completely inhibited by 3-amino-4-hydroxyphenyldichloroarsine hydrochloride, was completely restored on addition of glutathione (10 times the concentration of the inhibitor). It has been reported by Friedenwald and Herrmann (5) that tyramine oxidase inhibited by *p*-chloromercuribenzoate is only partially restored on addition of glutathione and that complete reactivation is reached in the presence of cyanide. Addition of cyanide diminishes the amount of *p*-chloromercuribenzoate present in the preparation owing to the formation of a complex compound between cyanide and *p*-chloromercuribenzoate; diminished inhibition will be the result of cyanide addition. In fact, Friedenwald and Herrmann report 85 per cent inhibition with 0.01 M *p*-chloromercuribenzoate (30 micromoles presumably in 3 cc. of fluid); in the presence of 0.005 M cyanide (1 mg. of KCN presumably in

TABLE IX

Effect of —SH Reagents on l-Glutamate Oxidase (Activating Protein or Dehydrogenase)

Enzyme (water extract of pig liver acetone powder; diphosphopyridine nucleotide (DPN) split off by acidification at pH 4.6; resuspended in 0.1 M phosphate buffer, pH 7.3) 1 cc.; DPN, 2 γ of 40 per cent purity in 0.3 cc.; *l*-glutamate, 0.25 M, 0.5 cc.; inhibitor or H_2O, 0.4 cc.; 0.1 M phosphate, pH 7.4, 1.8 cc.; 24.5°; rate of reduction measured at 3450 A, the substrate being added at 0 time.

Inhibitor	Concentration	Half reduction of DPN	Inhibition
	M	*min.*	*per cent*
None		1.88	
p-Chloromercuribenzoate	5×10^{-4}	∞	Complete
Iodoacetamide	5×10^{-3}	1.95	None
3-Amino-4-hydroxyphenyldichloroarsine hydrochloride	1×10^{-3}	∞	Complete

TABLE X

Reactivation of Sulfhydryl Enzymes

The conditions of the experiments were similar to those described in the preceding tables.

Enzyme	Inhibitor	Inhibition	Thiol	Reactivation
		per cent		*per cent*
Acetate oxidation, bakers' yeast	*p*-Chloromercuribenzoate, 1 5 $\times 10^{-3}$ M	Complete	Glutathione, 1 $\times 10^{-2}$ M	75
" " *Corynebacterium creatinovorans*	3-Amino-4-hydroxyphenyldichloroarsine hydrochloride, 2 $\times 10^{-4}$ M	98 5	" 4 $\times 10^{-3}$ "	91
β-Hydroxybutyrate oxidase	*p*-Chloromercuribenzoate, 5 $\times 10^{-4}$ M	Complete	" 5 $\times 10^{-3}$ "	Complete
Lipase (pancreatic)	3-Amino-4-hydroxyphenyldichloroarsine hydrochloride, 1 $\times 10^{-3}$ M	52	" 1 $\times 10^{-3}$ "	44 5
d-Amino acid oxidase	3-Amino-4-hydroxyphenyldichloroarsine hydrochloride, 1 $\times 10^{-3}$ M	91	" 1 $\times 10^{-3}$ "	Complete
Monoamine oxidase	*p*-Chloromercuribenzoate, 1 $\times 10^{-3}$ M	83	" 1 $\times 10^{-2}$ "	30
	p-Carbamylphenylarsine oxide, 1 $\times 10^{-3}$ M	76	" 1 $\times 10^{-2}$ "	Complete
	p-Aminophenyldichloroarsine hydrochloride, 1 $\times 10^{-3}$ M	77	" 1 $\times 10^{-2}$ "	"
Transaminase	*p*-Chloromercuribenzoate, 1 $\times 10^{-3}$ M	49	H_2S, 1 $\times 10^{-2}$ M	"
	" 1 $\times 10^{-3}$ "	49	Cysteine, 5 $\times 10^{-3}$ M	97
l-Glutamate oxidase	" 5 $\times 10^{-4}$ "	Complete	Glutathione, 5 $\times 10^{-3}$ M	69
	3-Amino-4-hydroxyphenyldichloroarsine hydrochloride, 1 $\times 10^{-3}$ M	"	" 1 $\times 10^{-2}$ "	Complete

3 cc. of fluid) the inhibitions dropped to 35 per cent. The "complete reactivation" on addition of glutathione is thus due to diminished inhibition. The tyramine oxidase preparation used by us, when inhibited by *p*-chloromercuribenzoate (83 per cent inhibition), was reactivated 30 per cent on addition of glutathione (ratio of glutathione to inhibitor, 10:1); on the other hand, the inhibition produced by 1×10^{-3} M *p*-carbamylphenylarsine oxide and 1×10^{-3} M *p*-aminophenyldichloroarsine hydrochloride was completely abolished on addition of glutathione at the usual ratio of 10:1. Transaminase, inhibited by *p*-chloromercuribenzoate, was completely reactivated on addition of H_2S or cysteine. The activity of *l*-glutamate dehydrogenase, inhibited by *p*-chloromercuribenzoate and 3-amino-4-hydroxyphenyldichloroarsine hydrochloride, was completely restored on addition of glutathione (Table X).

Rôle of Glutathione—Since the discovery of glutathione in 1921 by Hopkins and the establishment of its almost universal distribution in the intracellular fluid of animal tissues, many hypotheses have been formulated regarding the biological rôle of this tripeptide. It has been shown by other investigators, as well as in the experiments reported here and in the preceding paper (21), that glutathione produces reactivation of —SH enzymes when the —SH groups have been rendered ineffective by oxidation or by mercaptide formation. Thus, in the preceding paper (21), it was shown that sulfhydryl groups are essential for the activity of a large number of enzymes concerned with the metabolism of carbohydrate. Phosphorylases, oxidases, carboxylases, and decarboxylases required —SH groups for enzyme activity. We have presented in this paper observations on another large group of enzymes; namely, those concerned with the metabolism of fats and proteins, for the activity of which the presence of —SH groups in the enzyme protein molecule is required. If to these enzymes we add those found previously, the result is an indeed impressive array of sulfhydryl enzymes. In some cases (urease) the essential —SH groups (sluggish —SH groups) are more or less protected from the action of oxidizing agents; in others (muscle phosphorylase, pyruvate oxidase) the essential —SH groups are extremely labile, being oxidized by such sluggish oxidizing agents as molecular oxygen (freely reacting —SH groups). The continuous production of oxidizing agents in the cell will tend to inhibit the activity of the —SH enzymes. It is therefore reasonable to assume that the main rôle of glutathione in cellular systems is that of continuous reactivation of the —SH enzymes. Of course, the degree of activity of these enzymes is determined, among other factors, by the oxidation-reduction potential of the cell; *i.e.*, by the concentration of glutathione, of ascorbic acid, of oxidants, and of hydrogen ions, etc.

SUMMARY

Among the enzymes concerned with the metabolism of fats, the following have been found to require the presence of —SH groups in the activating protein: liver stearate oxidase, *Bacillus coli* stearate and oleate oxidase, β-hydroxybutyric dehydrogenase, acetate oxidase, and pancreatic lipase. The enzyme that oxidizes oleic acid in peanuts contained no essential —SH groups. Among the enzymes for protein metabolism, the following were found to be —SH enzymes: *d*-amino acid oxidase, transaminase, *l*-glutamic dehydrogenase, and monoamine oxidase. Diamine oxidase, pepsin, and trypsin contained no essential —SH groups The enzymes were inhibited by alkylating agents (iodoacetamide) and by mercaptide-forming agents (*p*-chloromercuribenzoic acid and organic arsenicals). Addition of glutathione at a ratio of 10:1 of inhibitor restored the activity of these enzymes. The rôle of glutathione in cellular activities is discussed on the basis of these experiments.

BIBLIOGRAPHY

1. Green, D. E., Dewan, J. G., and Leloir, L F , *Biochem J.*, **31,** 934 (1937).
2 Yosii, S , *J Biochem* , Japan, **26,** 39 (1937).
3 Konrad-Lang, O S A , *Z physiol Chem.*, **261,** 240 (1939)
4 Perlzweig, W , *Science*, **76,** 435 (1932)
5 Friedenwald, J S , and Herrmann, H , *J. Biol Chem* , **146,** 411 (1942)
6. Mazza, F. P , and Cimmino, A , *Boll Soc ital biol sper* , **8,** 531 (1933).
7. Lynen, F , *Ann. Chem* , **552,** 270 (1942), **554,** 40 (1943)
8. Dubos, R , and Miller, B F , *J Biol Chem* , **121,** 429 (1937).
9. Weinstein, S. S , and Wynne, A M., *J Biol. Chem* , **112,** 649 (1935–36).
10 Michaelis, L., and Schubert, M. P , *J Biol Chem* , **106,** 331 (1934)
11 Hellerman, L , *Physiol Rev* , **17,** 454 (1937)
12. Bersin, T , in Nord, F F , and Weidenhagen, R , Ergebnisse der Enzymforschung, Leipzig, **4,** 68 (1935)
13. Krebs, H. A., *Biochem J* , **29,** 1620 (1935)
14. Warburg, O , and Christian, W , *Biochem Z* , **298,** 150 (1938).
15. Negelein, E , and Bromel, H , *Biochem Z* , **300,** 225 (1939).
16. Cohen, P P , *J Biol Chem* , **136,** 565 (1940).
17. Cohen, P. P , *Biochem J.*, **33,** 551 (1939)
18 Blaschko, H , Richter, D , and Schlossmann, H , *Biochem. J.*, **31,** 2187 (1939).
19. Laskowski, M , *J Biol Chem* , **145,** 457 (1942)
20. Dewan, J G , *Biochem J* , **32,** 1386 (1938)
21 Barron, E S G , and Singer, T P., *J. Biol. Chem.*, **157,** 221 (1945).

IMPROVEMENTS IN THE SCHOENHEIMER-SPERRY METHOD FOR THE DETERMINATION OF FREE CHOLESTEROL*

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Owing to the increased demand for the determination of free cholesterol, the method of Schoenheimer and Sperry (1) was modified to develop a shorter, simpler, and yet reliable procedure suitable for clinical use.

EXPERIMENTAL

The experiments which led to the improvements (incorporated in our final method) in the procedure of Schoenheimer and Sperry are briefly described below.

As Kaye's (2) modification of the method of Drekter, Sobel, and Natelson (3) (hitherto used in our laboratory) was taken for comparison with the newly developed method, its reliability was confirmed by comparing it with the method of Schoenheimer and Sperry (1). Excellent agreement between the two methods is shown by values of 57.3, 57.7, 71.9, and 72.4 mg. of free cholesterol per 100 cc. of serum for Schoenheimer and Sperry's method and corresponding values of 57.3, 57.9, 72.4, and 72.4 for Kaye's method.

At first the serum was extracted by heating on a steam bath with the alcohol-acetone mixture, as described in the literature (1), but this step was found to be unnecessary. Extraction at room temperature sufficed equally well, as is seen in Table I. For the cold extraction the technique described in this paper was used with 5 cc. of alcohol-acetone mixture.

The next step was to find the minimum amount of alcohol-acetone mixture needed for the complete extraction of the free cholesterol in 0.2 cc. of serum. Table II shows the comparative results obtained when 5, 4, 3, and 2 cc. of alcohol-acetone mixture were used to extract 0.2 cc. of serum by the technique described in this paper. It is seen that good results are obtained with 3 cc. or more but not with 2 cc. Equally good results were obtained when the serum was extracted twice with 2 cc. of alcohol-acetone mixture. This, however, introduces an extra step that can be avoided except for the most exacting work.

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From this point on 3 cc. of alcohol-acetone mixture were used to extract 0.2 cc. of serum.

In all of the above experiments an aqueous digitonin solution, prepared according to Schoenheimer and Sperry (1), was used for the precipitation of free cholesterol. However, when aqueous digitonin was used the precipitate was sticky, the washing required a stirring rod, and the subsequent dissolving of the precipitate involved some difficulties, probably due to the insolubility of other lipids in 33 per cent aqueous solution. To overcome this the same experiments were carried out with a 50 per cent alcoholic solution of digitonin for the precipitation of free cholesterol.

TABLE I

Free Cholesterol Extracted at Refluxing and at Room Temperatures

The values are expressed as mg. per 100 cc of serum.

Refluxing temperature	Room temperature	Kaye's method
61.5	61.2	61.3
57.4	57.2	57.2
69.5	70.0	70.2
61.4	61.2	61.2

TABLE II

Influence of Volume of Extracting Fluids on Free Cholesterol Values

The values are expressed as mg. per 100 cc. of serum.

5 cc	4 cc	3 cc	2 cc	Kaye's method
59.7	60.1	60.3	58.6	60.3
61.5	61.2	61.2	60.5	61.3
69.5	69.8	70 0	68.2	70.2
73.6	73.1	73.3	70.2	73.8

This change in procedure eliminated the difficulties mentioned above. The stirring rod and washing with acetone-ether were abandoned. The washed precipitate dissolved with relative ease in acetic acid. The results compared favorably, as shown in Table III.

Up to this point the precipitation of free cholesterol took at least 16 hours at room temperature. The next experiments were carried out to find the minimum time required for complete precipitation at room temperature. Table IV shows the comparison between precipitations for 16 hours, 3 hours, and 1½ hours at room temperature. The precipitation was complete in 16 hours, but this process was neither complete nor were the results constant when the precipitation took place for 3 hours at room temperature.

To shorten the time required for complete precipitation the influence of temperature was investigated. The precipitation of the digitonide was carried out in the refrigerator, at room temperature, at 37°, and at 60°. As may be seen in Table V, at 37° precipitation was complete in 3 hours, while with decreasing temperature the precipitation is incomplete in 3 hours. In the final method the 3 hour period at 37° was adopted, since shorter periods proved less consistently reliable. It is possible, however, that with a more intensive investigation of temperatures an even shorter period for complete precipitation may be obtained.

TABLE III

Precipitation of Free Cholesterol with Alcoholic and Aqueous Digitonin

The values are expressed as mg. per 100 cc. of serum.

Aqueous digitonin	Alcoholic digitonin	Kaye's method
56.7	56.9	56.7
59.3	59.4	59.4
61.3	61.1	61.5
70.4	71.0	70.8

TABLE IV

Influence of Time on Completeness of Precipitation of Free Cholesterol at Room Temperature

The values are expressed as mg. per 100 cc of serum

About 16 hrs	3 hrs	1½ hrs	Kaye's method
85.5	80.1	69.3	85 2
90.0	81.6	66.7	89.8
43.0	38.8	30.9	42.6

TABLE V

Influence of Temperature on Precipitation of Free Cholesterol in 3 Hours

The values are expressed as mg. per 100 cc of serum

Refrigerator	Room temperature	37°	60°	Kaye's method
53.7	62.3	70.8	66.7	70.9
54.6	57.5	60.5	59.5	60 3
75.4	78.3	89.0	92.4	89.0

In all of the above experiments the color was developed as described in this paper and compared in the micro cups of a Bausch and Lomb visual colorimeter. All results in tables represent the average values of duplicates or triplicates.

stream, so as to produce a finely suspended precipitate which appears milky and homogeneous to the eye and is followed by the formation of visible protein particles within a minute. The precipitate is readily centrifuged and is well extracted. This technique favors completeness of extraction, because there is at least temporarily a homogeneous phase. With the usual techniques in which serum is added to solvent a layer of precipitate forms, outside of the drops of serum, which impedes penetration of the solvent. Mechanical agitation and heating are required to break up the clumps to permit full extraction.

The volume of the extraction fluid was kept as low as possible to enable the mixing of the reaction mixture without the aid of a stirring rod in a 15 cc. centrifuge tube. The volume recommended in our procedure is such as to provide a safety margin for the extracting of even large amounts of *free cholesterol* (see Table VIII, Samples 11 and 12).

TABLE VI

Influence of Volume of Extraction Fluid on Completeness of Extracting Total Cholesterol in 0.1 Cc. of Serum

The values are expressed as mg per 100 cc. of serum.

2 extractions and 1 rinsing		1 extraction and 1 rinsing		Kaye's method
Volume of solvent for each extraction				
1 cc.	2 cc	2 cc	3 cc	
156	181	143	182	182
222	237	187	237	237
290	304	260	305	303
250	268	218	266	268

These conditions, however, are insufficient to extract *total cholesterol* completely. A systematic study of this was made by extracting 0.1 cc. portions of serum with various amounts of solvent. The pertinent information is presented in Table VI. It is seen here that when one extraction and one washing by rinsing (see the technique above) were employed 2.0 cc. are insufficient. 3 cc., however, are sufficient. This is twice the ratio of solvent to serum employed for the free cholesterol estimation, for which twice as much serum is used for the same amount of solvent. With two extractions followed by one rinsing 1 cc. is insufficient for each extraction but 2 cc. are sufficient. Thus, here again the ratio of solvent to serum necessary for complete extraction is about twice as high. A similar trend was observed by Sperry[1] who found that when he increased the ratio of serum to solvent by a factor of 1.5 to 2.0 the total cholesterol values were sometimes a little low, while the free cholesterol values agreed with those obtained by his usual technique.

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[1] Sperry, W. M., personal communication.

The method employed for washing may be criticized as being incomplete on theoretical grounds. It is seen in Table VII, however, that the results obtained with one extraction and one rinsing are similar to those obtained with two extractions and one rinsing. Thus one extraction and one rinsing are sufficient for the quantitative extraction of free cholesterol.

Precipitation and Washing of Cholesterol Digitonide—There are several innovations here that helped in the elimination of the somewhat cumbersome stirring rod recommended by Schoenheimer and Sperry (1) the use of which was continued in the later modifications by Sperry (4, 5). (1) The volume of the precipitation mixture was reduced to make possible convenient mixing, by means of tapping, in the 15 cc. centrifuge tube. (2) The rapid blowing of the digitonin into the acetone-alcohol extract aids in the rapid formation of a finely divided cholesterol digitonide. (3) The use of a solution of digitonin in 50 per cent alcohol instead of aqueous digitonin prevents the formation of a sticky precipitate, which forms when aqueous digitonin is employed. The precipitate formed was easily suspended and washed. Because of the relatively anhydrous nature of the precipitate obtained with alcoholic digitonin solution, the washing with acetone-ether was no longer necessary and was omitted. The sticky nature of the precipitate formed with the aqueous digitonin solution was the main reason for the use of the stirring rods by Schoenheimer and Sperry.[1]

TABLE VII

Influence of Washing by Rinsing on Completeness of Extracting Free Cholesterol in 0.2 Cc. of Serum

The values are expressed as mg. per 100 cc. of serum.

1 extraction and 0 rinsing	1 extraction and 1 rinsing	2 extractions and 1 rinsing
59.0	61.1	61.1
59.1	60.9	60.9
67.0	68.6	68.8
57.8	59.2	59.0

In washing the digitonide precipitate care must be taken to shake the precipitate well in the washing fluid to get rid of impurities that would interfere with the subsequent development of the color. Otherwise, the color may become yellowish green and difficult to compare.

All our reagents were at room temperature and thus consistent results were obtained. However, when the room temperature was more than

28° or less than 24°, we employed a constant temperature bath, which is strongly recommended by Sperry[1] (4–6).

Stability of Color under Reading Conditions—The stability of the color intensity of the standard solution was checked in the following manner. The color of the standard was developed in the dark for 27 minutes, and then read in a Klett-Summerson photoelectric colorimeter with the red Filter 66. The liquid was continuously exposed to light. During the first 5 minutes no change took place, after 7 minutes there was a decrease of 0.8 per cent, after 9 minutes 1.3 per cent, after 10 minutes 1.7 per cent, and after 15 minutes 5.4 per cent. Thus the best time for matching the colors is the first 5 minutes after the 27 minute period of color development, although results obtained in the first 10 minutes are still satisfactory, since the unknown and standard change at about the same rate.

TABLE VIII

Comparison between Kaye's and Authors' Methods

The values are expressed as mg per 100 cc of serum. Each value is the average of two duplicates.

Sample No	New method	Kaye's method	Sample No	New method	Kaye's method
1	56.7	56.7	7	61.3	61.5
2	59.3	59.3	8	61.5	61.5
3	59.3	59.6	9	72.7	72.6
4	59.7	59.8	10	93.1	92.7
5	61.2	61.2	11	172.0	172.0
6	61.2	61.5	12	184.0	182.0

Total Cholesterol—This method of free cholesterol estimation may be advantageously used in conjunction with the total cholesterol method recently published by Sperry and Brand (6), which is relatively simple but gives results that compare favorably with the more involved digitonin method of Schoenheimer and Sperry (1), and its later improvements by Sperry (4, 5).

Results

Table VIII shows twelve representative results obtained by the method described in this paper compared with Kaye's (2) method, which was previously used in our laboratory. Each value is the average of two determinations. Attention is called to the close agreement of the high values found in Samples 11 and 12.

The mean value of forty estimations (each value being the average of duplicates) by the new method was 65.6 mg. per cent; the mean value of forty estimations in duplicate by Kaye's method was 65.4 mg. per cent

The average difference was 0.2 mg. per cent. The maximum difference was 1.0 mg. per cent in one case.

The mean values and standard deviations of known amounts of cholesterol added to the cholesterol extract of blood serum are given below.

No of determinations	Cholesterol added	Mean amount recovered	Average deviation from mean	Standard deviation of mean
	mg	*mg*	*mg.*	*mg*
24	0.100	0.099	±0.0018	±0.0020

The observation of Sperry (7) that in normal persons the ratio of free to total cholesterol is constant is confirmed by the data in Table IX, in which each value is the average of two determinations. Our values for the ratio are from 22.6 to 29.2 per cent, which is similar to those obtained by Kaye (2) whose total cholesterol method we employed. The values of Sperry are from 24.3 to 30.1 per cent. The difference is probably due to the fact that our total cholesterol values, for which no saponification was employed, are higher than his.

TABLE IX

Values of Normal Individuals

The values are expressed as mg per 100 cc. of serum.

Sample No	Total cholesterol	Free cholesterol	Free cholesterol	Sample No	Total cholesterol	Free cholesterol	Free cholesterol
	mg	*mg*	*per cent*		*mg*	*mg*	*per cent*
1	266	60.4	22.6	11	255	67.5	26.5
2	202	47.1	23.3	12	225	59.7	26.6
3	251	59.6	23.8	13	241	64.7	27.0
4	213	49.2	24.3	14	165	45.4	27.5
5	248	60.8	24.5	15	247	67.5	27.5
6	248	60.8	24.5	16	238	63.8	27.9
7	267	66.0	24.7	17	209	59.5	28.5
8	267	66.6	24.9	18	179	51.1	28.6
9	215	54.2	25.2	19	255	73.2	28.7
10	160	41.5	25.9	20	208	60.4	29.2
					Range 160–267	Range 41–73	Range 22.6–29.2

SUMMARY

Certain improvements in the technique of the method of Schoenheimer and Sperry for estimation of free cholesterol in blood serum are described. The advantages gained are that the whole sample of serum is used, the stirring rod is eliminated, the precipitation is completed within 3 hours

at 37°, and the precipitate is easily washed with ether and dissolved in glacial acetic acid. The ratio between free and total cholesterol is constant in normal cases.

We are indebted to Dr. W. M. Sperry of the New York State Psychiatric Institute for submitting the results of his unpublished experiments to the authors on the estimation of cholesterol.

BIBLIOGRAPHY

1. Schoenheimer, R., and Sperry, W. M., *J. Biol. Chem.*, **106**, 745 (1934).
2. Kaye, I. A., *J. Lab. and Clin. Med*, **25**, 996 (1940).
3. Drekter, I. J., Sobel, A. E., and Natelson, S., *J. Biol. Chem.*, **115**, 391 (1936).
4. Sperry, W. M., *Am. J. Clin. Path., Tech. Suppl.*, **2**, 91 (1938).
5. Sperry, W. M., The Schoenheimer-Sperry method for the determination of cholesterol, New York (1943).
6. Sperry, W M., and Brand, F C., *J. Biol. Chem*, **150**, 315 (1943).
7. Sperry, W. M., *J. Biol. Chem.*, **114**, 125 (1936).

A LACTOBACILLUS ASSAY METHOD FOR *l*(+)-GLUTAMIC ACID*

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Several investigators have discussed the essentiality of *l*(+)-glutamic acid for the growth of *Lactobacillus arabinosus* 17-5 (1–7). This paper presents a quantitative method for the determination of *l*(+)-glutamic acid based upon its ability to stimulate growth of this organism, as measured by the production of lactic acid, in a medium containing the known requirements with the exception of *l*(+)-glutamic acid. The techniques are based for the most part on those previously described for the microbiological determination of vitamins and amino acids by the use of *Lactobacillus arabinosus* 17-5 (1, 3, 4, 6–11).

The microbiological method appears to have the following advantages over methods previously described for the determination of glutamic acid: It is more precise; it distinguishes between the optical isomers; it can be used with materials containing relatively small amounts of *l*(+)-glutamic acid; and it requires only a few mg. for analysis.

The relatively extensive amino acid requirements of the *Lactobacilli* used in vitamin assays are usually supplied by casein hydrolysates, but for assays of amino acids other than tryptophane it has been necessary to use mixtures of purified amino acids. However, we have found that a casein hydrolysate freed from glutamic acid by the method described below is suitable for the assay of *l*(+)-glutamic acid. Apart from the advantage of lower cost, the casein hydrolysate may supply amino acids not yet recognized as either essential or stimulating to the growth of *Lactobacillus arabinosus* 17-5.

Glutamic acid was removed from casein hydrolysates by conversion to pyrrolidonecarboxylic acid, followed by extraction with ethyl acetate. The procedure was repeated as often as necessary to reduce the glutamic acid content to a negligible amount. The work of previous investigators on the glutamic acid to pyrrolidonecarboxylic acid transformation has recently been reviewed (12). Olcott (12) showed that the reaction can be achieved to the extent of 92 to 95 per cent by autoclaving at 125° for 4 hours at an initial pH of 3.3, conditions which were slight modifications of those recommended by Wilson and Cannan (13). Other amino acids, with the exception of cystine, were not affected. Wilson and Cannan indicated that ethyl acetate is a satisfactory solvent for pyrrolidonecarboxylic acid at pH 1, while Pucher and Vickery (14) used ethyl acetate to extract the pyrrolidonecarboxylic acid formed by the hydrolysis of glutamine from plant extracts at pH 2.4. In the present investigation, the protein hydrolysate was adjusted to pH 2.9 to 3.0, for both cyclization and extraction with ethyl acetate.

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EXPERIMENTAL

Microorganism—The stock culture of *Lactobacillus arabinosus* 17-5[1] was carried in yeast extract-glucose-agar stab cultures. The reference stock culture was transferred monthly, incubated at 30° for 24 hours, and then stored in a refrigerator.

The medium used for the growth of inoculum was prepared as described below, but with ordinary casein hydrolysate (containing glutamic acid). The organism was transferred to the liquid subculturing medium from a fresh stab culture and incubated for 20 hours at 30°. The cells were centrifuged and resuspended in sterile 0.9 per cent sodium chloride solution.

Basal Medium—The composition of the basal assay medium is given in Table I. The constituents of the medium were prepared as follows:

Glutamic Acid-Free Casein Hydrolysate—500 gm. of technical casein were refluxed for 12 hours with 5 liters of concentrated hydrochloric acid. As much as possible of the hydrochloric acid was removed by repeated evaporations to a thick paste *in vacuo*. The hydrolysate was diluted to 1500 ml., filtered, adjusted to pH 2.9 with 15 N sodium hydroxide (200 ml. were required), and autoclaved for 4 hours at 125°. The autoclaved solution, pH 2.7, was filtered, placed in a continuous liquid-liquid extractor, and extracted for 48 hours with ethyl acetate. During one run some tyrosine separated during this step. At the end of the period, the solution was again at pH 2.9 as a result of removal of pyrrolidonecarboxylic acid. The autoclaving and extraction were repeated until the glutamic acid content had been so diminished as not to interfere with the microbiological assay. This required four or five complete cycles, followed by an additional autoclaving.

The rate of removal of pyrrolidonecarboxylic acid was estimated by determination of the amino nitrogen content (Van Slyke manometric method) of the ethyl acetate-soluble fractions before and after reconversion of the pyrrolidonecarboxylic acid to glutamic acid by acid hydrolysis. In one run, the following amounts were removed in four successive fractionations: 43, 22, 13, and 7 gm. Since the conversion of glutamic acid to pyrrolidonecarboxylic acid at pH 2.9 should be 90 to 95 per cent complete with each autoclaving (12, 13), it appears that extraction with ethyl acetate is the less efficient step in the procedure. Determination of the free amino nitrogen content of these extracts before hydrolysis permitted an estimate of the amounts of unknown amino acids extracted simultaneously. These were approximately 4, 4, 4, and 6 gm., respectively.

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[1] No. 8014, American Type Culture Collection, Georgetown University Medical School, Washington, D. C.

The 1500 ml. of casein hydrolysate solution remaining contained per ml. approximately 33 mg. of nitrogen, including 5 mg. of ammonia nitrogen, and 100 mg. of sodium chloride. Thus, 70 per cent of the nitrogen of the casein was still present. For convenient use the solution was diluted to give 16 mg. of nitrogen per ml., and stored at room temperature.

TABLE I

Composition of Basal Medium

	per cent		*p p m.*
Glucose	1.0	Adenine	10
Glutamic acid-free acid-hydrolyzed casein	0.5	Guanine	10
		Uracil	10
l-Cystine	0.01	Thiamine hydrochloride	0.5
l-Tryptophane	0.01	Riboflavin	0.2
Sodium acetate trihydrate	1.0	Calcium pantothenate	0.1
" chloride	1.0	Nicotinic acid	0.5
Potassium monohydrogen phosphate trihydrate	0.05	Pyridoxine hydrochloride	0.5
		Biotin	0.01
Potassium dihydrogen phosphate	0.05	*p*-Aminobenzoic acid	0.01
Magnesium sulfate heptahydrate	0.02		
Ferrous sulfate heptahydrate	0.001		
Manganous sulfate tetrahydrate	0.001		

The absence of peptides or anhydrides was indicated by the unchanged amino nitrogen content of an aliquot that was autoclaved with 20 per cent hydrochloric acid for 4 hours at 125°.

Although the rate of removal of pyrrolidonecarboxylic acid as given above indicated the possible presence of as much as 0.05 per cent of *l*(+)-glutamic acid in the casein hydrolysate solution after the final autoclaving at pH 2.9, evidence to be presented below showed that the hydrolysate was substantially free of *l*(+)-glutamic acid (less than 0.005 per cent). For convenience, it will be referred to as "glutamic acid-free casein hydrolysate."

l-Cystine—5 gm. of *l*-cystine were dissolved in 10 ml. of concentrated hydrochloric acid, and diluted to 1 liter.

l-Tryptophane—2.5 gm. of *l*-tryptophane were dissolved in 500 ml. of water with warming. The solution was stored in a refrigerator.

Adenine, Guanine, and Uracil—A solution containing 1 mg. per ml. of each of these substances was prepared as follows: 100 mg. of uracil, 124 mg. of guanine hydrochloride, and 174 mg. of adenine sulfate were suspended in a small volume of water; 2 ml. of concentrated hydrochloric acid were added; the mixture was heated to complete solution, cooled, and diluted to 100 ml.

Vitamin Supplement—A solution was made up to contain 5 mg. of thiamine hydrochloride, 2 mg. of riboflavin, 1 mg. of calcium pantothenate, 5 mg. of nicotinic acid, 5 mg. of pyridoxine hydrochloride, 100 γ of biotin, and 100 γ of *p*-aminobenzoic acid per 100 ml. Crystalline vitamins were used with the exception of biotin, which was supplied as a concentrate (S. M. A. Corporation, No. 1000 or 5000). The solution was stored in a refrigerator.

Inorganic Salts—Solution A contained 25 gm. each of potassium monohydrogen phosphate trihydrate and potassium dihydrogen phosphate in 250 ml. of water. Solution B contained 10 gm. of magnesium sulfate heptahydrate and 0.5 gm. each of sodium chloride, ferrous sulfate heptahydrate, and manganous sulfate tetrahydrate in 250 ml. of water. A few drops of concentrated hydrochloric acid were added to Solution B to prevent precipitation.

Procedure

The basal medium was mixed immediately prior to use. To prepare, for example, 1 liter of solution (enough for 200 tubes), 20 gm. each of glucose, sodium acetate trihydrate, and sodium chloride were dissolved in 768 ml. of water. To this solution were added 100 ml. of glutamic acid-free hydrolyzed casein solution (1600 mg. of nitrogen),[2] 40 ml. of *l*-tryptophane solution, 20 ml. of *l*-cystine solution, 20 ml. of adenine-guanine-uracil mixture, 20 ml. of vitamin supplement, and 10 ml. each of inorganic Solutions A and B. The medium was adjusted to pH 6.8 to 6.9 with approximately 12 ml. of 4 N sodium hydroxide.

The basal medium was pipetted in 5 ml. portions into Pyrex culture tubes (18 × 150 mm.). Appropriate aliquots of the standard *l*(+)-glutamic acid solution and of the solutions to be assayed were next added and the total volume in each tube was made to 10 ml. with distilled water. The following amounts of *l*(+)-glutamic acid were found to be convenient

[2] If tyrocidine is lost during the preparation of glutamic acid-free casein hydrolysate, the addition to the basal medium of 1 mg. per tube is recommended, since small amounts exerted some stimulation when added to a basal medium low in tyrosine.

for establishing the standard curve: 130, 150, 170, 200, 250, 300, and 400 γ per tube. Assay samples were chosen to contain approximately 150, 200, and 250 γ of *l*(+)-glutamic acid. For precise work, both standard and sample were diluted so that 5 ml. aliquots could be added to the culture tubes. Each level was run in quadruplicate.

The racks of tubes were slanted and shaken until the basal medium and test materials were thoroughly mixed. The tubes were then plugged with cotton and autoclaved at 15 pounds of steam pressure for 10 to 15 minutes, allowed to cool, and inoculated.

Since the amount of acid produced was found to depend upon the amount of inoculum, it was necessary that this be kept as constant as possible from tube to tube. A 1:7 saline resuspension[3] of a 20 hour subculture was dispensed from a hypodermic syringe equipped with a 22 gage needle and held at a fixed angle. 3 drops (approximately 0.06 ml.) were introduced into each tube.

The tubes were inoculated so that if half of any set of replicates was inoculated toward the start the other half would be inoculated toward the end. By this means it was easy to recognize occasional progressive changes in the level of inoculation, such as might have resulted, for example, from sedimentation of the bacterial suspension.

The tubes were incubated at 30° ± 0.5° for 64 hours and then autoclaved to stop acid production. The cultures were titrated with 0.1 N sodium hydroxide with bromothymol blue indicator. For precise work, the pH of the titrated solution was measured (glass electrode) and a correction of 0.01 ml. of 0.1 N sodium hydroxide was made for each 0.02 pH unit of difference from pH 6.90.

Assay values were calculated from a standard curve obtained by plotting ml. of acid produced against micrograms of *l*(+)-glutamic acid supplied. A new standard curve was established for each assay experiment. Values calculated for different aliquots of the sample were averaged to give the final assay. Any trend of assay value with size of aliquot was taken as evidence of the presence of interfering factors, and substances giving such trends required special investigation.

Preparation of Samples for Assay—The method was applied to proteins, polypeptides, yeast, and Steffen's waste (a technical source of *l*(+)-glutamic acid (15)). Except where otherwise indicated, the samples were hydrolyzed by being refluxed with 20 per cent hydrochloric acid on an oil bath (120–125°) for 24 hours, as previously described (12). Before use, they were neutralized with sodium hydroxide.

Recovery of Added l(+)-Glutamic Acid—The amount of added *l*(+)-

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[3] The number of cells varied around 250,000,000 per ml. We are indebted to Doris Hirschmann for direct microscopic counts.

glutamic acid recoverable in the presence of protein hydrolysates was determined by several procedures. In some cases the l(+)-glutamic acid was added to the sample either before or after hydrolysis and the results were compared with parallel assays of similar samples prepared without added l(+)-glutamic acid. In other cases the recovery was tested by the following procedure. The major portion of l(+)-glutamic acid in the hydrolysate was converted to pyrrolidonecarboxylic acid, after which a calculated equivalent amount of l(+)-glutamic acid was restored to the hydrolysate. The assay of such a restored hydrolysate was compared with the assay of the original hydrolysate. It will be convenient to refer to these experiments as "indirect recoveries." They are of particular interest inasmuch as the amino acids of the sample, with the exception of glutamic acid, were present in approximately the same amounts as in the original assay.

In all cases values are reported as the percentage recoverable of the total l(+)-glutamic acid present. The latter value was calculated from the amount of l(+)-glutamic acid added and from independent assays of the unknown.

DISCUSSION

Basal Media with Mixtures of Amino Acids—The use of crystalline amino acids in the basal medium for l(+)-glutamic acid assay, although more expensive than the use of glutamic acid-free casein hydrolysate, may be preferable when assays of a limited number of samples for several amino acids are contemplated. Shankman (6) described basal media supporting good growth of *Lactobacillus arabinosus* 17-5 in which the amino acid requirements were supplied by crystalline amino acids, and Shankman, Dunn, and Rubin (7) obtained an accurate assay value for l(+)-glutamic acid in a mixture of crystalline amino acids.

In attempts to use Shankman's amino acid mixture, Medium *b*, for the assay of l(+)-glutamic acid in hydrolysates of casein, gelatin, and gliadin, we obtained unreasonably high assay values, and recoveries of added l(+)-glutamic acid ranged from 115 to 150 per cent. It was later found that assays and recoveries comparable to those observed with the basal medium of glutamic acid-free casein could be obtained if a mixture of crystalline amino acids approximating the composition of casein (without l(+)-glutamic acid) was used. The synthetic casein hydrolysate differed from Shankman's amino acid mixture, Medium *b*, principally in its content of *l*-proline, *l*-hydroxyproline, and *dl*-serine, and in its markedly higher content of *l*-tyrosine. The primary deficient factor (for accurate l(+)-glutamic acid assays of protein hydrolysates) of Shankman's mixture was *l*-proline, although the addition of all four amino acids gave better results than the addition of *l*-proline alone. Experimental data are given in Fig. 1 and in Table II.

Other Variations in Basal Medium—The optimum amount of glutamic acid-free casein hydrolysate was chosen on the basis of the data shown in Fig. 2. 8 mg. of nitrogen (as hydrolysate) permitted maximum acid production and approximately maximum expression of the initial plateau in the standard curve. As will be shown later, this plateau appears to be due in large part to the aspartic acid and arginine in the casein hydrolysate.

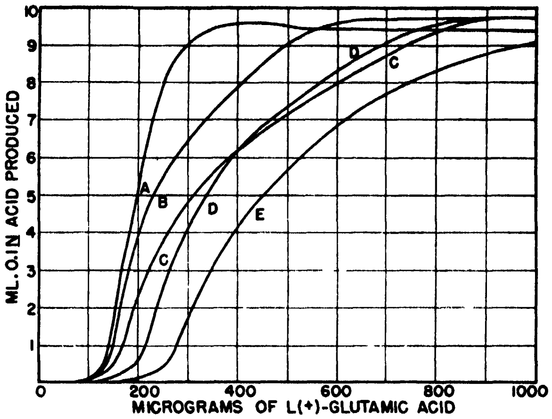

FIG. 1. Response of *Lactobacillus arabinosus* 17-5 to $l(+)$-glutamic acid on media prepared with various amino acid supplements. The following sources of amino acids were used (see Table III for a further description): Curve A, glutamic acid-free casein hydrolysate plus *l*-cystine and *l*-tryptophane; Curve B, Shankman's (6) mixture plus *l*-proline, *dl*-serine, *l*-hydroxyproline, and *l*-tyrosine; Curve C, Shankman's mixture plus *l*-proline; Curve D, Shankman's mixture plus *dl*-serine, *l*-hydroxyproline, and *l*-tyrosine; Curve E, Shankman's mixture unsupplemented.

It is desirable that enough glutamic acid-free casein hydrolysate be used in the basal medium so that errors resulting from the presence of these substances in assay samples will be small.

The effect of sodium chloride was investigated, since considerable amounts are present in protein hydrolysates after neutralization of the hydrochloric acid used for hydrolysis. The addition of 40 and of 200 mg. of sodium chloride per tube to a basal medium which contained only about 40 mg. per tube of sodium chloride from the glutamic acid-free casein hydrolysate stimulated acid production in the lower part of the standard curve equivalent to a 3 and 6 per cent increase respectively of added $l(+)$-glutamic acid. The effect disappeared at levels of acid production approaching the maximum. 100 mg. of sodium chloride per assay tube

TABLE II

l(+)-Glutamic Acid Assays with Various Basal Media

Hydrolysate	Sources of amino acids in basal medium*					
	Glutamic acid-free casein hydrolysate + l-cystine and l-tryptophane		Shankman's amino acid mixture†		Shankman's mixture† + l-proline, dl-serine, l-hydroxyproline, and l-tyrosine‡	
	l(+)-Glutamic acid	Recovery§	l(+)-Glutamic acid	Recovery§	l(+)-Glutamic acid	Recovery§
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Gliadin	43.9	100	67.9	124	45.7	100
Casein	20.4	101	35.2	112	20.4	101
Gelatin	10.2	100	16.7	123	10.5	103

* Ingredients other than the amino acids as in Table I

† Without l(+)-glutamic acid. *dl*-Threonine 2 mg., *l*-leucine 2 mg., *dl*-isoleucine 2 mg., *dl*-valine 2 mg., *dl*-methionine 1 mg., *l*-cystine 1 mg., *l*-tryptophane 0.33 mg., *l*-tyrosine 0.33 mg., *dl*-phenylalanine 1 mg., *l*-lysine 2 mg., *dl*-alanine 2 mg., *l*-arginine 0.5 mg., *l*-aspartic acid 4 mg., *l*-histidine 0.5 mg. per tube

‡ *l*-Proline 4.5 mg., *dl*-serine 3.7 mg., *l*-hydroxyproline 5 mg., and *l*-tyrosine 2 mg. per tube.

§ Approximately equal amounts of l(+)-glutamic acid as standard and as protein hydrolysate were mixed and assayed, and the amount found was compared with the total amount calculated to be present from simultaneous assay of the protein hydrolysate

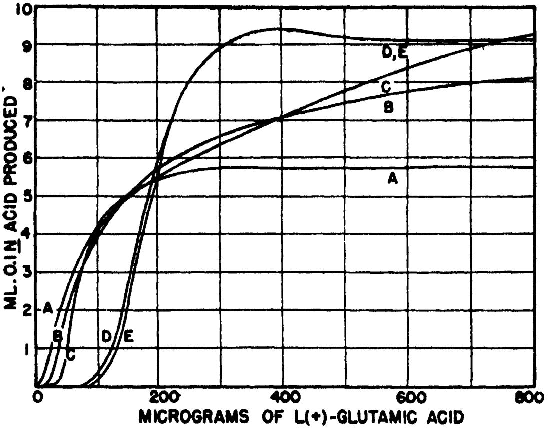

FIG. 2. Response of *Lactobacillus arabinosus* 17-5 to l(+)-glutamic acid as affected by the amount of glutamic acid-free casein hydrolysate supplied. All other constituents of the medium were kept constant. Glutamic acid-free casein hydrolysate was supplied to give the following amounts of nitrogen per tube: Curve A, 0.24 mg.; Curve B, 0.8 mg.; Curve C, 2.4 mg.; Curve D, 8 mg.; Curve E, 10.4 mg.

were therefore added to the basal medium, so that the addition of sodium chloride in any ordinary sample of a protein hydrolysate would have negligible effect.

Ammonium chloride had an effect equivalent to less than 2 γ of $l(+)$-glutamic acid per mg. of ammonia; hence, the amounts of ammonia ordinarily present in protein hydrolysates would not be expected to affect assay values. The basal medium contained 16 per cent of the nitrogen of the glutamic acid-free casein hydrolysate as ammonium ion. If a synthetic mixture of amino acids is used, it may be desirable to add an ammonium salt (approximately 1 mg. per tube) to the basal medium.

Standard Curve—When the response of acid production was plotted against the amount of $l(+)$-glutamic acid added, sigmoidal curves were obtained. The position and to some extent the form depended upon the time and temperature of incubation, the level of inoculation, and the composition of the basal medium.[4] The standard curve differed from those obtained with most *Lactobacillus* assays, in which there are no initial plateaus. It also differed from the sigmoidal standard curve obtained in the *Lactobacillus* assay for *p*-aminobenzoic acid (9), which does not undergo the marked shift in sensitivity with time of incubation or amount of inoculum.

The effect of length of incubation period on the standard curve is shown in Fig. 3. Relatively small amounts of $l(+)$-glutamic acid could be measured when long incubation periods were used, while for short incubation periods much larger amounts of $l(+)$-glutamic acid were required to permit appreciable growth and acid production.

Although such a relationship between the quantitative requirement for an essential growth factor and the time of incubation has not been reported previously, within the authors' knowledge, the phenomenon is reminiscent of the relationship between the minimum inhibitory dose of many antibacterial agents and the time of incubation. Whatever the mechanism of the biological reaction may be, the reproducibility and specificity of the response of *Lactobacillus arabinosus* 17-5 make this organism suitable for the determination of *l*(+)-glutamic acid.

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[4] Incubation temperatures of 25°, 30°, and 35° gave progressively shorter initial plateaus in the standard curves, after 3 and 4 days of incubation time, the displacements of the standard curve corresponded to 5 to 10 γ of $l(+)$-glutamic acid per degree of difference of temperature from 30° Variation in the amount of inoculum had a similar effect A 3-fold increase of the prescribed inoculation level reduced the length of the initial plateau (3 days of incubation) by about 30 γ of $l(+)$-glutamic acid. Small variations in the temperature of the medium at the time of inoculation did not have a perceptible effect; assays of standard $l(+)$-glutamic acid in which the temperature of the medium at the time of inoculation had been held at 18°, 25°, and 35° agreed within 0 3 per cent. In a test for homogeneity of the stock culture of *Lactobacillus arabinosus*, substantially identical standard curves were obtained with cultures derived from sixteen separate colonies picked from dilution plates of the stock organism

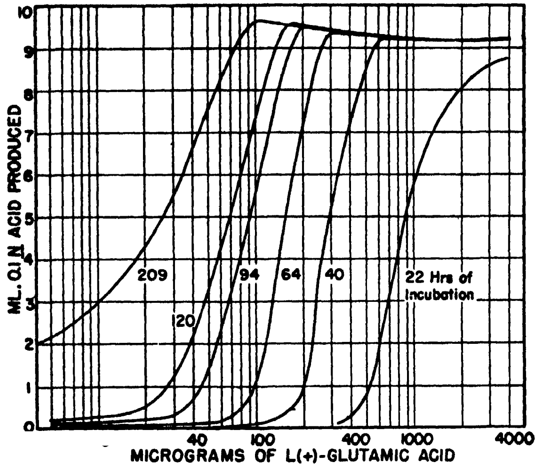

FIG. 3. The effect of period of incubation on the response of *Lactobacillus arabinosus* 17-5 to *l*(+)-glutamic acid. The amount of inoculum was about twice that generally used.

TABLE III

Effect of Period of Incubation on Assay of Casein Hydrolysate

Period of incubation	Approximate *l*(+)-glutamic acid test levels	*l*(+)-Glutamic acid content of casein	Recovery of *l*(+)-glutamic acid*
hrs.	γ	*per cent*	*per cent*
22	600	18.9	100.0
	1000	18.7	102.7
	1400	18.3	109.1
40	250, 400	19.7	100.4
60	140, 200, 250	20.0	100.4
94	100, 140	20.1	99.7
120	40, 60, 80	19.5	98.5
209	10	18.7	98.5
	20	19.0	98.5
	40	19.3	98.9

* Approximately equal amounts of *l*(+)-glutamic acid were supplied as casein hydrolysate and as standard *l*(+)-glutamic acid added to the hydrolysate.

Assay and recovery data for a casein hydrolysate were obtained simultaneously with the standard curves of Fig. 3 (Table III). 2 to 4 days of incubation gave maximum assay values and accuracy. Values obtained with the shortest and with the longest incubation periods were lower than the maximum values by 5 per cent or more. Definite trends of assay values with the size of the aliquot were observed in both cases, and markedly divergent recoveries of added *l*(+)-glutamic acid in the former. The recovery value obtained for the 1400 γ aliquot at 22 hours is particularly interesting since it is high, while the assay value is low, an observation which emphasizes the errors possible in correction factors based upon recovery values.

It was possible to estimate residual *l*(+)-glutamic acid in the glutamic acid-free casein hydrolysate used in the basal medium from data such as are given in Fig. 3. If the dosage-response data for 209 hours are plotted with *linear* coordinates, extrapolation shows that the basal medium contained about 7 γ of *l*(+)-glutamic acid per tube in excess of that required for the initial plateau of the standard curve, since the basal medium without added *l*(+)-glutamic acid permitted production of 1.32 ml. of 0.1 N acid per tube in this time. If the amount of *l*(+)-glutamic acid required for the initial plateau of the standard curve is plotted against the period of incubation, it may be estimated that the plateau at 209 hours was negligible. The *l*(+)-glutamic acid content of the medium was accordingly in the neighborhood of 7 γ per tube, demonstrating the effectiveness of the procedure for preparing glutamic acid-free casein hydrolysate.

Specificity—The ability to replace the specific growth promotion rôle of *l*(+)-glutamic acid was tested for a number of compounds related to glutamic acid or containing glutamic acid residues (Table IV). *dl*-Glutamic acid was found to be more than 50 per cent active, in contrast to the observation of Kuiken *et al.* (3). Pure *d*(−)-glutamic acid was about 8 per cent as active as *l*(+)-glutamic acid in the absence of *l*(+)-glutamic acid, and 10 per cent as active in the presence of *l*(+)-glutamic acid. The activity of α-ketoglutaric acid was small but significant. α-Hydroxyglutaric acid was inactive, as were all derivatives in which the amino group was blocked.

Pollack and Lindner (5) found that *l*-glutamine was essentially as active as *l*(+)-glutamic acid for *Lactobacillus arabinosus* 17-5. At the level tested in this study, *l*-glutamine was 140 per cent as active as *l*(+)-glutamic acid (Table IV). Although preliminary observations indicate that this relationship is variable, the point is not important for purposes of assay, since *l*-glutamine is converted quantitatively to *l*(+)-glutamic acid during acid hydrolysis.

Of the three peptides of *l*(+)-glutamic acid tested, *l*-glutamyl-*l*-tyrosine, in which the α-carboxyl group of the glutamic acid residue is blocked, had very low activity. *l*-Glutamyl-*l*-glutamic acid had up to 24 per cent activity. Glutathione, in which the ω-carboxyl groups of the glutamic acid residues are blocked whereas the amino and α-carboxyl groups are free, had much higher activity.

TABLE IV

Specificity of Response of Lactobacillus arabinosus to l(+)-Glutamic Acid

Substance*	Quantity tested per tube	Basal level of l(+)-glutamic acid per tube	Activity per glutamic acid residue
	γ	γ	*per cent*
l(+)-Glutamic acid, recrystallized	150–250	0	100.0 (Assigned)
" " reagent grade	150–250	0	99.7
d(−)-Glutamic acid	3000–5000	0	7.2–8.4
" "	200, 1000	150	7.6, 8.0
" "	200, 1000	250	9.8, 11.0
dl-Glutamic acid	300–500	0	54.5–55.7
l-Glutamine	200	0	140†
α-Ketoglutaric acid	1000	150	6.3
" "	5000	150	1.6
α-Hydroxyglutaric acid	1000, 5000	150, 250	<0.2
l-Pyrrolidonecarboxylic acid	1000, 5000	150, 250	<0.2
Carbobenzoxy-l-glutamic acid	1000, 5000	150, 250	<0.2
p-Aminobenzoyl-l-glutamic acid	1000, 5000	150, 250	<0.2
p-Nitrobenzoyl-l-glutamic "	1000, 5000	150, 250	<0.2
l-Glutamyl-l-glutamic acid	2000	0	∼10
" "	80, 400	150, 250	17–24
l-Glutamyl-l-tyrosine	1000	150, 250	2.5
"	5000	150, 250	1.8
Glutathione	300	0	94
"	400	0	78
"	500	0	69

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* We wish to acknowledge our indebtedness to E. F. Jansen for l-glutamine; D. L. Shemin for α-ketoglutaric acid; H. Fraenkel-Conrat for l-glutamyl-l-glutamic acid and carbobenzoxy-l-glutamic acid; M. Bergmann for l-glutamyl-l-tyrosine. Pure d(−)-glutamic acid, $[\alpha]_D^{24}$ in 1.73 N hydrochloric acid, −31.7°, was obtained by several crystallizations as the hydrochloride from acid hydrolysates of the polypeptide produced by a specific strain of *Bacillus subtilis* (16). We are indebted to M. S. Dunn for determining the rotation of this preparation. A microbiological assay of a hydrolysate of the polypeptide disclosed the presence of about 15 per cent of l(+)-glutamic acid (corrected for the activity of the d(−)-glutamic acid present). Synthetic dl-glutamic acid was from Amino Acid Manufactures. l-Pyrrolidonecarboxylic acid, α-hydroxyglutaric acid, p-aminobenzoyl-l-glutamic acid, and p-nitrobenzoyl-l-glutamic acid were synthesized by recognized methods.

† l-Glutamine was sterilized with ether, since large losses were found when it was autoclaved in the neutral basal medium. The activity of l(+)-glutamic acid was substantially identical whether it was sterilized with ether or by autoclaving in the basal medium.

A number of amino acids were tested individually by addition of each to the basal medium of glutamic acid-free casein hydrolysate supplemented with 150 and 250 γ of l(+)-glutamic acid per tube. 10 mg. of any one of the following amino acids per tube had no or only a small stimulatory effect, varying in magnitude up to that obtained with 20 γ of *l*(+)-glutamic acid: *dl*-alanine, *dl*-α-amino-*n*-valeric acid, *l*-cystine, glycine, *l*-hydroxyproline, *dl*-isoleucine, *dl*-isovaline, *l*-leucine, *dl*-lysine, *dl*-methionine, *dl*-norleucine, *dl*-phenylalanine, *l*-proline, *dl*-serine, *dl*-threonine, *l*-tyrosine, and *dl*-valine. In those amino acids not of synthetic manufacture, it is possible that the slight stimulatory effects might have been due to the presence of *l*(+)-glutamic acid in amounts of 0.2 per cent or less.

l-Ornithine, choline, and *d*-glucosamine, when tested in the same way, possessed activities per mg. equivalent to 15, 12, and 5 γ, respectively, of *l*(+)-glutamic acid. No effect could be demonstrated with 100 γ of choline or with 10 mg. of betaine.

A further group of amino acids, when tested at convenient levels varying from 1 to 10 mg. per tube, gave the following depressive effects expressed as micrograms of *l*(+)-glutamic acid equivalent per mg. of amino acid: *l*-aspartic acid, −40, *l*-arginine, −15; *l*-asparagine, −4; *dl*-α-aminobutyric acid, −4, *l*-tryptophane, −3, and *l*-histidine, −2. The depressive effects of *l*-aspartic acid and *l*-arginine were great enough to account for the initial plateau of standard curves obtained on basal media containing glutamic acid-free casein hydrolysate.

These effects were of approximately the same magnitude when tested at points high and low on the standard curve (250 and 150 γ of *l*(+)-glutamic acid per tube), and resulted in virtually linear displacements of the standard curve. Moreover, when various combinations of the more active amino acids were tested, the resultant effects were the sums of the effects of the amino acids taken individually; *i.e.*, no synergistic actions were evident.

The relatively small effects of both stimulatory and depressive amino acids were obtained with amounts equal to or larger than the amounts supplied to the basal medium by the glutamic acid-free casein hydrolysate. The results thus show that the basal medium is essentially optimal with respect to essential or stimulatory amino acids. From the standpoint of the accurate determination of *l*(+)-glutamic acid, the basal medium is essentially optimal with respect to depressive amino acids also. Thus if the most active interfering amino acid, *l*-aspartic acid, was present in the sample in amounts equal to that of glutamic acid, the resulting effect would give values for *l*(+)-glutamic acid low by about 4 per cent. Since glutamic acid is present in most proteins in much larger amounts than aspartic acid, it seems that in these cases such a source of error may be neglected.

Racemization, of l(+)-Glutamic Acid—The extent of racemization of *l*(+)-glutamic acid during hydrolysis was evaluated as follows: A sample of *l*(+)-glutamic acid refluxed for 72 hours with 20 per cent hydrochloric acid was found to possess 95.4 per cent of the biological activity of pure *l*(+)-glutamic acid. An unheated control sample showed no change in activity. The 4.6 per cent loss of biological activity after 72 hours of treatment with hot acid is comparable to that observed by others (17–19) for the racemization of *l*(+)-glutamic acid, as measured by optical methods.

It appears evident that assays of *l*(+)-glutamic acid are low by approximately 1.5 per cent for each 24 hours of acid hydrolysis under our conditions, if it is assumed that the major portion of the *l*(+)-glutamic acid of the sample is present in the free state during most of the hydrolysis period (*cf.* Table V, 24 and 48 hour hydrolysis periods).

l(+)-Glutamic acid heated in 20 per cent sodium hydroxide for 72 hours on an oil bath at 120° lost 33 per cent of its original activity.

Effect of Period of Hydrolysis on Assays of Casein—The effects of various periods of hydrolysis in boiling 20 per cent hydrochloric acid on *l*(+)-glutamic acid assays of casein are shown in Table V. Data for three of the periods are presented in detail to illustrate a change of trend in assay values. After 6 hours of hydrolysis, the trend had substantially disappeared and the average recovery varied from 99.5 per cent to 101.5 per cent. The magnitudes of the assay values were high for short hydrolysis periods and became progressively lower with longer hydrolysis. This decrease was almost complete with 24 hours of hydrolysis, although a further slight drop attributable to racemization was noted with 48 hours of hydrolysis. Olcott (12) found that the hydrolysis of casein was essentially complete after 24 hours of hydrolysis.

For hydrolysis periods shorter than 6 hours, when marked trends were apparent, the recovery was calculated for individual levels of casein, assayed with and without added *l*(+)-glutamic acid. Recovery values obtained in this way, although somewhat variable, averaged about 100 per cent, indicating the additive nature of the effects of (added) free *l*(+)-glutamic acid, and of *l*(+)-glutamic acid isotels[5] in the short period hydrolysates. If this additive effect holds for the smaller aliquots of short period casein hydrolysates plus *l*(+)-glutamic acid, and one assumes 100 per cent recovery of added *l*(+)-glutamic acid, it is possible to obtain values for smaller aliquots of hydrolysate than could be assayed directly because of the sigmoidal nature of the standard curve. Such values extend in a regular manner the trend toward increasingly higher assay values for smaller aliquots of short period casein hydrolysates. Values obtained in this way are shown in parentheses in Table V.

The effects of very small amounts of short period casein hydrolysates on the standard *l*(+)-glutamic acid assay curve were determined. For

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[5] Compounds related by their common ability to perform the same function (20).

TABLE V

*Effect of Length of Period of Hydrolysis and Size of Aliquot on l(+)-Glutamic Acid Assays of Casein**

Hydrolysis period	Experiment	Aliquot tested		*l*(+)-Glutamic acid found	Calculated *l*(+)-glutamic acid content of casein*	Recovery of *l*(+)-glutamic acid
		Casein*	Added *l*(+)-glutamic acid			
hrs.		*mg*	γ	γ	*per cent*	*per cent of calculated value*†
0.5	A	0.25	50	185	(54.0)‡	
		0.375	75	225	(40.0)	
		0.50		188	37.6	
		0.50	100	282	(36.4)	97.9
		0.625	125	338	(34.1)	102.9
		0.75		219	29.2	
		1.00		255	25.5	
		1.25		295	23.6	
1	"	0.25 -0.625§	50-125		(50.8)-(34.1)	104.5, 95.6
		0.50 -1.25			37.2 - 23.4	
2	"	0.25 -0.625	50-125		(36.8)-(29.1)	91.3, 105.2
		0.50 -1.25			32.0 - 23.8	
3	"	0.375-0.625	75-125		(24.8)-(23.5)	104.3
		0.75 -1.25			23.7 - 21.4	
3	B	0.375-0.625	75-125		(24.0)-(22.9)	102.2
		0.75 -1.25			23.2 - 22.4	
4	A	0.375	75	166	(24.3)	
		0.50	100	215	(23.0)	
		0.625	125	267	(22.7)	100.4
		0.75		168	22.4	
		1.00		212	21.2	
		1.25		263	21.0	
6	B	0.375-0.625	75-125		(20.6) Average	101.5 Average
		0.75 -1.25			20.0 "	
12	"	0.375-0.625	75-125		(19.1) "	99.5 Average
		0.75 -1.25			19.3 "	
24	"	0.375-0.625	75-125		(18.8) "	101.0 Average
		0.75 -1.25			18.4 "	
48	"	0.375	75	143	(18.2)	100.4
		0.50	100	194	(18.8)	101.9
		0.625	125	242	(18.7)	101.6
					(18.6) Average	101.3 Average
		0.75		136	18.1	
		1.00		181	18.1	
		1.25		227	18.2	
					18.1 Average	

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* Air-dry; 9.8 per cent moisture.

† It was assumed that the assay of casein hydrolysate plus added *l*(+)-glutamic acid was equal to the sum of separate assays of equal aliquots of each constituent.

TABLE V—*Concluded*

‡ Values in parentheses are calculated on the assumption that 100 per cent of the added l(+)-glutamic acid was recovered. The smaller aliquots of casein did not possess sufficient activity to fall on the standard curve unless l(+)-glutamic acid was added.

§ In the interest of economy of space, only the extreme values for series corresponding to those presented in detail for 0.5, 4, and 48 hours are given for the other periods of hydrolysis.

example, 250, 100, 50, and 25 γ per tube of casein hydrolyzed for ½ hour gave displacements of the standard curve equivalent to 113, 66, 41, and 24 γ of l(+)-glutamic acid, respectively. The last figure represents an apparent l(+)-glutamic acid assay value of 96 per cent for casein hydrolyzed for ½ hour, or approximately 5 times the value for casein hydrolyzed for 24 hours. These results indicate the presence in short period casein hydrolysates of very active isotels of l(+)-glutamic acid, which show proportionately greater effects when added in small amounts.

Although three peptides containing l(+)-glutamic acid were shown to be less active than the amino acid, the protein component responsible for the unexpected stimulation of growth by the partial hydrolysate of casein may be an unidentified peptide. The necessity for complete hydrolysis prior to assay is emphasized by the results of these experiments.

Precision—The response of *Lactobacillus arabinosus* 17-5 to l(+)-glutamic acid has been shown to be highly reproducible within a given experiment, perhaps surprisingly so in view of the shifting nature of the dosage-response curve. Replicate titrations have shown standard deviations of 0.06 ml. of 0.1 N acid when corresponding to the ascending portion of the standard curve, and of 0.02 ml. when corresponding to the initial or final plateau. Reproducibility of this sort would give a standard error of less than 0.2 per cent for an assay value based on twelve tubes, if a theoretically correct standard curve is assumed. In practice, however, interpolation between the points ordinarily used to establish the standard curve may lead to errors of 1 per cent or larger in magnitude, particularly for regions of inflection.

l(+)-Glutamic Acid Assays of Proteins and Other Substances—l(+)-Glutamic acid assays of a number of proteins, and of yeast, Steffen's waste, and tyrocidine hydrochloride are presented in Table VI. Assays of most of these materials for total glutamic acid by Olcott's (12) method are also given. Similarly prepared hydrolysates of the same lots of materials were used in both types of assays.

For most proteins the agreement between the two methods is fairly close, although the assay values obtained by the microbiological method tend to be lower. These results suggest (*a*) that the values obtained by the

TABLE VI

l(+)-*Glutamic Acid Contents of Proteins and Other Materials**

All the assays have been run on dried materials or have been calculated to the dry basis.

Substance	$l(+)$-Glutamic acid content by microbiological assay	Recovery of $l(+)$-glutamic acid added†		Hydrolysate autoclaved 4 hrs., at 120°, initial pH 3.3			Indirect recovery of added $l(+)$-glutamic acid	Total glutamic acid content by chemical assay (Olcott (12))
		Before hydrolysis	After hydrolysis	Residual $l(+)$-glutamic acid		Recovery of added $l(+)$-glutamic acid†		
	per cent	*per cent*	*per cent*	*per cent of protein*	*per cent of original $l(+)$-glutamic acid*	*per cent*	*per cent*	*per cent*
β-Lactoglobulin, crystalline	18.7		100.3	0.83	4.4	102.3	101.0	21.5
Edestin	19.1		100.0					18.3
Zinc insulin	17.5		100.2					19.6
Tobacco mosaic virus	12.5‡		98.5‡					17.0
Lysozyme	3.4‡		101.4					4.0
Silk fibroin	2.1		100.6					3.5
Gliadin	44.2		100.3	2.62	5.9	100.8	101.8	45.7
" §	44.0		98.2					
Gluten	32.2		100.8	1.96	6.1	102.3	101.0	35.0
Glutenin	36.7		101.1					35.9
Gelatin, technical	10.2		100.3	0.31	3.0	100.1	101.4	12.0
" " §	10.8		102.0					
Casein, "	19.7		100.7	1.10	5.6	101.4	100.8	22.0
" " + 11.10% $l(+)$-glutamic acid	30.7	99.7	100.8	1.82	6.0	100.9	100.8	
Zein, technical	24.8		100.7					23.5
Fibrin	12.4		100.2					16.0
Growth hormone§	14.5		99.5					
Lactogenic hormone§	13.4		100.1					
Purothionine§	2.7		99.5					
Tyrocidine hydrochloride	8.3‡		98.4‡					12.0
Torula yeast	8.0		101.1	0.50	6.2	102.0	100.5	
" " + 8.00% $l(+)$-glutamic acid	15.7	98.2	99.9	1.16	7.4	100.4	101.2	
Steffen's waste	15.9		100.8	0.86	8.6	99.5	101.0	
" " + 14.25% $l(+)$-glutamic acid	29.9	99.0	100.8	1.60	8.6	101.8	101.6	
Pepsin, crystalline	11.5‡		107.2‡				97.8	12.3
Egg albumin, crystalline	12.1		94.8	0.44	3.7	89.7	89.9	17.0
" " " 72 hrs. hydrolysis	11.6		95.5	0.35	3.0	87.4	96.8	
Egg albumin, crystalline,§ modified assay conditions	13.7		98.3				102.0	
Egg albumin, crystalline, + 15.00% $l(+)$-glutamic acid	34.9	92.1	97.7	1.20	4.8	94.0	95.3	

TABLE VI—*Concluded*

* We are indebted to the following for samples of proteins and other materials: E. F. Jansen for a solution of five times recrystallized β-lactoglobulin; H. P. Lundgren for a solution of recrystallized egg albumin; D. M. Greenberg for edestin; H. C. Reitz for crystalline pepsin; Eli Lilly and Company for crystalline zinc insulin; W. M. Stanley for tobacco mosaic virus prepared by differential centrifugation; C. H. Li for growth hormone and lactogenic hormone; A. K. Balls for purothionine; G. Alderton for lysozyme; E. F. Jansen and K. P. Dimick for tyrocidine hydrochloride; Amino Products Company for Steffen's waste.

† Approximately equal amounts of *l*(+)-glutamic acid were supplied as sample hydrolysate and as standard *l*(+)-glutamic acid added to the hydrolysate.

‡ The following trends of assay values were found with increasing size of sample aliquot from 150 to 250 γ of *l*(+)-glutamic acid; 20 per cent decrease for pepsin assay and direct recovery but no trend for the indirect recovery; 5 per cent decrease for tobacco mosaic virus and tyrocidine hydrochloride assays, and 2.5 per cent for direct recoveries; and 8 per cent decrease for lysozyme assay. Small trends of doubtful significance were noticed in some other cases, particularly in assaying residual *l*(+)-glutamic acid after autoclaving at pH 3.3.

§ The assay conditions were modified to increase sensitivity by tripling the amount of inoculum and by incubating at 34° The assay range at 64 hours of incubation extended from 20 to 150 γ of *l*(+)-glutamic acid per tube.

chemical method of assay may be high (12), (*b*) that the rate of racemization of *l*(+)-glutamic acid in the protein structure is greater before than after it has been liberated, or (*c*) that *d*(−)-glutamic acid occurs in appreciable amounts in certain proteins. For tobacco mosaic virus and tyrocidine hydrochloride the microbiological assays were lower than those obtained by the chemical method by about 25 per cent; these materials appear to deserve more detailed investigation. The microbiological values for β-lactoglobulin and edestin are somewhat lower than the maximum reported values obtained by isolation (21); however, there is no information available concerning the optical rotation of this total isolated glutamic acid for β-lactoglobulin. That given for edestin indicates that 9.6 per cent was present as the *d* form.

Recovery experiments were made to test the accuracy of the *l*(+)-glutamic acid assays. With a few exceptions to be discussed later these recoveries varied from 98 to 102 per cent. Recoveries of *l*(+)-glutamic acid added before hydrolysis appeared to fall 1 or 2 per cent lower than recoveries of *l*(+)-glutamic acid added after hydrolysis, undoubtedly a result of a moderate degree of racemization.

The residual *l*(+)-glutamic acid in samples autoclaved at pH 3.3 was found to vary from 3 to 8 per cent of the amount originally present.[6]

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[6] Olcott (12) used a correction of 8 per cent for the unconverted glutamic acid remaining after 4 hours of autoclaving at an initial pH of 3.3 and 22 pounds of steam pressure. The results obtained during this investigation indicate that this factor may be variable, and somewhat less than 8 per cent (Table VI, fifth column).

Recovery experiments on these converted hydrolysates showed but slightly greater deviations from the theoretical than those on the original hydrolysates. Since the aliquots in assays for residual *l*(+)-glutamic acid in converted hydrolysates contained 12 to 20 times the amounts of amino acids, other than glutamic acid, used in the original assays, it is clear that errors due to the presence of these other amino acids must have been very small.

In two cases discrepancies in recovery experiments or trends of assay value with size of sample indicated the presence of interfering substances. Thus, crystalline pepsin gave assay values approximately 20 per cent higher for 150 γ of *l*(+)-glutamic acid than for 250 γ aliquots, while the recovery of *l*(+)-glutamic acid added after hydrolysis was significantly (7 per cent) high. Crystalline egg albumin likewise gave anomalous recovery values, although in this case no trend of assay value was observed. The anomalous behavior of egg albumin was unchanged by an increase in the period of hydrolysis to 72 hours. It was made less evident, however, by the use of a higher incubation temperature and a larger amount of inoculum, which permitted the assay of smaller amounts of egg albumin.

No systematic attempt has been made to study the anomalies in the assay of egg albumin or pepsin. Although corrections based on recovery experiments might be made, the validity of such corrections has not been established and their use would necessarily result in loss of precision. Preliminary observations suggest that the disturbing influences might be eliminated by the addition to the basal assay medium of sufficiently large amounts of glutamic acid-free hydrolysates of the materials showing the anomalous results. The method of preparing glutamic acid-free casein hydrolysate described above is applicable to other proteins. It is also possible that the interfering substances in egg albumin or pepsin might be eliminated by a preliminary quantitative separation of the dicarboxylic acids by a procedure such as has been suggested by Cannan (22). Such a separation might also prove useful in increasing the precision of Olcott's (12) method for total glutamic acid.

In the assay of new materials it would appear to be desirable to run parallel assays with added *l*(+)-glutamic acid. With the precision attained in this study, a deviation of 2 per cent from the theoretical recovery would serve to indicate the presence of a disturbing factor giving rise to errors of at least that degree of magnitude in the assay. There may be, however, sources of error that would not give rise to significant departures from theoretical recoveries (see Table V).

Comparison of Total and l(+)-Glutamic Acid Content in Normal and Tumor Tissue.—Kögl and his coworkers (23) have recently reaffirmed their contested belief that malignant tissues contain appreciable amounts of unnatural amino acids, particularly of $d(-)$-glutamic acid. It appeared that evidence bearing on this controversy could be readily obtained by a comparison of the amounts of total glutamic acid (12) and $l(+)$-glutamic acid in normal and malignant tissues. The proteins of a Brown-Pearce rabbit testicular carcinoma and of normal rabbit testes were obtained by extraction of the minced tissues with 95 per cent alcohol and with 5 per cent trichloroacetic acid in a Waring blendor. The results of analyses are shown in Table VII. They indicate that the abnormal tissue contained no more $d(-)$-glutamic acid than did normal tissue, at least as determined by these methods.

TABLE VII

*Comparison of $l(+)$- and Total Glutamic Acid Contents of Proteins from Rabbit Testicular Brown-Pearce Carcinoma and Normal Rabbit Testes**

Source of protein†	$l(+)$-Glutamic acid		Total glutamic acid‡
	Assay	Recovery	
	per cent	*per cent*	*per cent*
Brown-Pearce carcinoma	10.0	100.0	14
Normal tissue	9.8	99.2	13

* We are indebted to J. W Thompson of the National Cancer Institute for samples of these tissues.

† Both samples contained 14.0 per cent nitrogen (dry weight).

‡ As determined by Olcott's method (12). Corrected for cystine content. The differences between the two methods of assay are similar to those observed with other proteins containing 10 to 15 per cent glutamic acid.

We are grateful to D. K. Mecham, Alice L. McGilvery, and Evelyn McCombs of this Laboratory for helpful technical assistance.

SUMMARY

Lactobacillus arabinosus 17-5 was used for the quantitative determination of $l(+)$-glutamic acid. The amino acid requirements other than $l(+)$-glutamic acid were supplied by a glutamic acid-free casein hydrolysate, prepared by repeated cycles of autoclaving at pH 3 to convert glutamic acid to pyrrolidonecarboxylic acid and selective extraction of the latter with ethyl acetate. The effects of a number of factors involved in the assay were studied in detail. This biological assay method is capable of a precision of 1 to 2 per cent.

Assays have been made of acid hydrolysates of proteins, polypeptides, yeast, and Steffen's waste. For most hydrolysates, the accuracy of the assays as judged by recovery experiments approached the precision. Of

some twenty materials assayed only hydrolysates of crystalline egg albumin and crystalline pepsin contained interfering substances which gave recovery values in error by as much as 5 to 10 per cent.

The $l(+)$-glutamic acid contents of the following proteins are reported for the first time: silk fibroin, 2.1 per cent;[7] lysozyme, 3.5 per cent; purothionine, 2.7 per cent; growth hormone, 14.5 per cent; lactogenic hormone, 13.4 per cent.

BIBLIOGRAPHY

1. Hegsted, D. M., *J. Biol. Chem.*, **152,** 193 (1944).
2. Hutchings, B. L., and Peterson, W. H , *Proc. Soc. Exp. Biol and Med.*, **52,** 36 (1943).
3. Kuiken, K. A., Norman, W. H., Lyman, C. M., and Hale, F., *Science*, **98,** 266 (1943).
4. Kuiken, K. A., Norman, W. H , Lyman, C M., Hale, F., and Blotter, L., *J. Biol. Chem* , **151,** 615 (1943).
5. Pollack, M. A , and Lindner, M , *J. Biol Chem* , **143,** 655 (1942)
6. Shankman, S., *J. Biol. Chem* , **150,** 305 (1943).
7. Shankman, S , Dunn, M S., and Rubin, L B , *J. Biol. Chem* , **150,** 477 (1943).
8. Greene, R. D., and Black, A., *Proc. Soc. Exp. Biol. and Med.*, **54,** 322 (1943).
9. Lewis, J. C., *J. Biol. Chem.*, **146,** 441 (1942)
10. McMahan, J. R., and Snell, E. E., *J. Biol. Chem.*, **152,** 83 (1944).
11. Snell, E. E., and Wright, L. D , *J Biol. Chem.*, **139,** 675 (1941).
12. Olcott, H. S., *J Biol. Chem* , **153,** 71 (1944).
13. Wilson, H., and Cannan, R. K , *J. Biol. Chem* , **119,** 309 (1937).
14. Pucher, G. W., and Vickery, H B., *Ind. and Eng. Chem., Anal. Ed.*, **12,** 27 (1940).
15. O'Day, D. W., and Bartow, E , *Ind and Eng. Chem.*, **35,** 1152 (1943).
16. Bovarnick, M., *J. Biol. Chem.*, **145,** 415 (1942).
17. Arnow, L. E., and Opsahl, J. C., *J. Biol. Chem.*, **133,** 765 (1940).
18. Behrens, O. K., Lipmann, F., Cohn, M., and Burk, D., *Science*, **92,** 32 (1940).
19. Johnson, J. M., *J. Biol. Chem.*, **134,** 459 (1940).
20. Williams, R. J., *Science*, **98,** 386 (1943).
21. Chibnall, A. C., Rees, M W., and Williams, E. F., *Biochem. J.*, **37,** 372 (1943).
22. Cannan, R. K., *J. Biol. Chem.*, **152,** 401 (1944)
23. Kögl, F., Erxleben, H., and van Veersen, G. J., *Z. physiol. Chem.*, **277,** 251 (1943).
24. Dunn, M. S., Camien, M. N., Rockland, L. B., Shankman, S., and Goldberg, S. C., *J. Biol. Chem.*, **155,** 591 (1944).

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[7] Since this paper was submitted, Dunn *et al.* (24) have reported that silk fibroin contains 2.16 per cent glutamic acid, microbiologically assayed.

A SIMPLE GRAVIMETRIC METHOD FOR THE DETERMINATION OF URINARY POTASSIUM

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The method to be described involves the precipitation of potassium as the silver cobaltinitrite after the interfering substances in urine are eliminated by a dry ashing in the presence of excess sulfuric acid.

Precipitation of potassium as the silver cobaltinitrite was described by Burgess and Kamm (1) and utilized by Breh and Gaebler (2) in the estimation of serum potassium. The constancy of composition of the potassium silver cobaltinitrite has been questioned, although recent studies (3–5) indicate that for amounts of potassium usually contained in blood serum, a precipitate of constant composition can be obtained if certain precautions are observed.

In the present method precipitation is initiated at 18–20° which is reported to be optimal for obtaining a precipitate of constant composition (4, 5). The precipitate is collected, dried, and weighed in a sintered glass filter as employed in the determination of sodium according to Butler and Tuthill (6).

The method is simple, permits reasonably accurate results with a wide range of urinary potassium concentration, and can be carried out with ordinary laboratory equipment. It compares favorably with the standard chloroplatinate method in accuracy and has advantages over this method in being simpler, more rapid, and less expensive.

Reagents and Glassware—

1. *Sodium cobaltinitrite reagent.* This reagent is prepared as described in Peters and Van Slyke's book of methods (7) and is stored in a brown bottle in the refrigerator. The solution deteriorates 3 to 4 weeks after preparation and may give high results if used after this time.

2. *40 per cent silver nitrate.* 40 gm. of silver nitrate dissolved in distilled water and made up to 100 ml.

3. *Silver cobaltinitrite reagent.* This reagent is prepared fresh at the time of analysis. 1 ml. of 40 per cent silver nitrate is added for each 10 ml. of sodium cobaltinitrite solution. The mixture is shaken thoroughly to redissolve the precipitate formed. It is filtered through coarse filter paper after standing for 10 minutes.

4. *10 N H_2SO_4.* 280 ml. of concentrated C.P. acid diluted to 1 liter with distilled water.

5. *Sintered glass filters.* These filters should be of No. 4 porosity, have a capacity of approximately 30 ml., and be suitable for weighing. After each determination these filters were cleaned with hot water, followed by heating on a steam bath immersed in 1:1 nitric acid, then washed on a suction filter with distilled water, 95 per cent ethyl alcohol, and lastly with ethyl ether. The carefully dried filter was stored in a vacuum desiccator over sodium hydroxide pellets. It was found necessary to weigh the filters before each determination as they consistently lose weight if adequately cleaned. The weighed filter was stoppered tightly from the bottom and placed in a 100 ml. beaker, resting on the stopper.

6. *Evaporating dishes.*[1] These were made of a Pyrex glass with a relatively high silica content such as is suitable for dry ashing and were of 30 to 50 ml. capacity.

Method

The urines used in this study were freshly voided or 24 hour specimens preserved with 10 ml. of 10 N H_2SO_4. 10 ml. of urine plus 2 ml. of 10 N H_2SO_4 were evaporated to a syrupy consistency on a steam bath, then placed in a cold muffle furnace and ashed overnight at a temperature ranging from 510–540°. After cooling, the ash was dissolved in distilled water with the aid of slight warming on the steam bath, quantitatively transferred to a 50 ml. volumetric flask, and made up to volume with distilled water. After thorough mixing, approximately 25 ml. of the ash solution were filtered through No. 40 Whatman paper.

The filtered ash solutions and the freshly prepared silver cobaltinitrite reagent were brought to a temperature of 18–20° by means of a water bath. 10 ml. of the reagent were pipetted into a weighed, stoppered filter followed by 10 ml. of the filtered ash solution which were added slowly. The mixture was stirred for 1 minute, covered with a watch-glass, and allowed to stand at room temperature. At the end of half an hour the stopper was removed and the precipitate filtered and washed with the aid of suction. Three 10 ml. portions of distilled water, two 10 ml. portions of 95 per cent alcohol, and two 10 ml. portions of ethyl ether were used in this order. When the precipitate was dry the filter was carefully removed from the suction flask, wiped dry on the outside, and placed in a vacuum desiccator. After 30 minutes, the precipitate was weighed to four decimal places on an analytical balance.

DISCUSSION

The weight of precipitate was determined for amounts of potassium ranging from 1.0 to 20.0 mg. (Table I). A standard solution of potassium

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[1] Vycor or vitreosil dishes are suitable for this purpose.

TABLE I

Recovery of Potassium from Standard Solutions by Direct Precipitation with Ag Cobaltinitrite

Amount of K	Weight of ppt.	Average weight of ppt.	Calculated per cent K in ppt.
mg.	*mg.*	*mg.*	
1.0	8.6		11.63
	8.8		11.36
	8.8		11.36
	9.0		11.11
	8.8		11.36
	8.1		12.35
	9.2	8.76 ± 0.33*	10.87
2.0	17.4		11.49
	17.3		11.56
	16.2		12.35
	17.3		11.56
	17.9		11.17
	17.3	17.23 ± 0.3	11.56
3.0	26.4		11.36
	25.8		11.63
	26.1		11.49
	25.9		11.58
	27.1		11.07
	26.3	26.27 ± 0.41	11.41
4.0	34.4		11.63
	34.2		11.70
	35.0		11.43
	34.4		11.63
	33.1		12.08
	33.8	34.15 ± 0.59	11.83
8.0	67.5		11.85
	69.1		11.58
	69.0		11.59
	69.3		11.54
	69.1		11.58
	68.0	68.67 ± 0.67	11.76
12.0	102.9		11.66
	102.9		11.66
	101.0		11.88
	101.4		11.83
	101.1		11.87
	99.0	101.38 ± 1.33	12.12
16.0	135.6		11.80
	135.6		11.80
	139.0		11.51
	138.4		11.56
	135.3		11.83
	136.5	136.73 ± 1.38	11.72

TABLE I—*Concluded*

Amount of K	Weight of ppt	Average weight of ppt	Calculated per cent K in ppt
mg.	*mg.*	*mg.*	
20.0	167.7		11.93
	167.9		11.91
	168.7		11.86
	164.1		12.19
	166.2		12.03
	168.1	167.12 ± 1.43	11.90
Mean.			11.66
Probable error			±0.28
(" " of mean $= \frac{0.6745\sigma}{\sqrt{N}}$)			

* Standard deviation from the mean, $\sigma = \sqrt{\Sigma d^2/N}$.

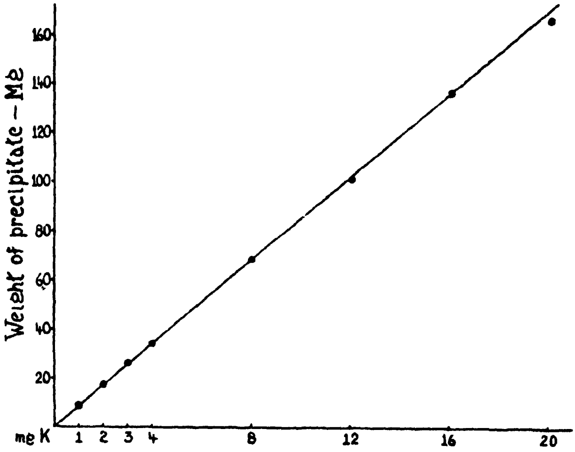

FIG. 1. Mean weights of precipitates for different amounts of potassium (six to seven determinations at each level) (see Table I).

sulfate was used. Fig. 1 shows that the weight of precipitate is proportional to the amount of potassium used, at least through the range of 1.0 to 16.0 mg. In the determination of a factor for use in calculation it was found that in general the proportion of potassium to precipitate increases slightly as the amount of potassium is increased. This fact necessitates the use of special factors for aliquots which are low (1.0 mg.) or high (16 to 20 mg.) in potassium, if a high degree of accuracy is desired. The mean factor of 0.1166 appears suitable for the estimation of urinary potassium under most circumstances.

In Table II are shown the results of determining the potassium content of standard solutions after evaporation and ashing as described for urine.

TABLE II

Recovery of Potassium from Ashed Potassium Sulfate Solutions (Factor = 0.1166)

Maximum temperature of ashing	Total amount of K ashed	Theoretical K content of aliquot used for determination	Calculated amount of K recovered	Per cent recovery	Average per cent recovery
°C.	*mg*	*mg.*	*mg.*		
540	8.0	1.6	1.64	102.5	
			1.63	101.9	
			1.47	91.9	
			1.53	95.6	
			1.71	106.9	
			1.66	103.8	
			1.62	101.3	
			1.54	96.3	100
727	8.0	1.6	1.55	96.9	
			1.47	91.9	
			1.59	99.4	
			1.48	92.5	
			1.50	93.8	
			1.40	87.5	
			1.48	92.5	93.5
540	20.0	4.0	4.07	101.8	
			4.05	101.3	
			4.06	101.5	
			4.10	102.5	101.8
727	20.0	4.0	3.93	98.3	
			3.92	98.0	
			3.96	99.0	
			3.92	98.0	98.3
540	40.0	8.0	7.98	99.8	
			8.19	102.4	
			8.01	100.1	
			7.89	98.6	
			7.86	98.3	
			8.08	101.0	
			7.81	97.6	
			7.96	99.5	
			8.00	100.0	99.7
727	40.0	8.0	7.86	98.3	
			7.82	97.8	
			7.78	97.3	
			7.82	97.8	97.8

The recovery values, in general, were within 2 per cent of the theoretical. Table II also shows that the recovery was lower when a higher temperature (727°) was used for ashing.

TABLE III

Gravimetric Chloroplatinate and Silver Cobaltinitrite Methods Compared

	K in aliquot	Determined K		Per cent recovery	
		Chloroplatinate	Ag cobaltinitrite	Chloroplatinate	Ag cobaltinitrite
Standard K_2SO_4 solution					
	mg	*mg*	*mg.*		
	8.0	7.83	7.85	97.9	98.1
	8.0	7.86	7.95	98.3	99.4
	8.0		8.10		101.2
	8.0		8.05		100.6
Average		7.85	7.99	98.1	99.8
Standard K_2SO_4 solution with added elements					
Na* 16.0	8.0	7.88	8.17	98.5	102.1
" 32.0	8.0	7.93	7.95	99.1	99.4
P 1.0 + Na 1.5	8.0	7.88	8.06	98.5	100.8
Ca 0.4 + Mg 0.4	8.0	7.81	8.00	98.4	100.0
Average		7.88	8.05	98.6	100.6
Urine					
		5.45	5.43		
		5.47	5.61		
		5.45	5.63		
		5.51	5.61		
Average		5.47	5.57		
Recovery of K added to urine					
	4.0		4.03		100.8
	4.0		4.09		102.2
	4.0		4.13		103.2
	4.0		4.09		102.2
Average			4.09		102.1
	4.0	4.04	4.03	101.0	100.8
	4.0	4.01	3.89	100.3	97.3
	4.0	4.00	3.93	100.0	98.3
	4.0	4.13	4.07	103.3	101.8
	4.0	3.98	4.11	99.5	102.5
	4.0	3.86	4.08	96.5	102.0
	4.0	3.83	3.76	95.8	94.0
	4.0	3.91	4.06	97.8	101.5

TABLE III—*Concluded*

	K in aliquot	Determined K		Per cent recovery	
		Chloroplatinate	Ag cobaltinitrite	Chloroplatinate	Ag cobaltinitrite
	mg	*mg*	*mg*		
	4.0	3.93	4.01	98.3	100.3
	4.0	3.93	3.94	98.3	98.5
	4.0	3.97	4.04	99.3	101.0
	4.0		4.03		100.8
Average		3.96	4.00	99.1	99.9

* Added as Na_2SO_4, $Na_2HPO_4 \cdot 12H_2O$, $CaSO_4$, $MgSO_4$, respectively; the amounts represent mg. in the aliquot.

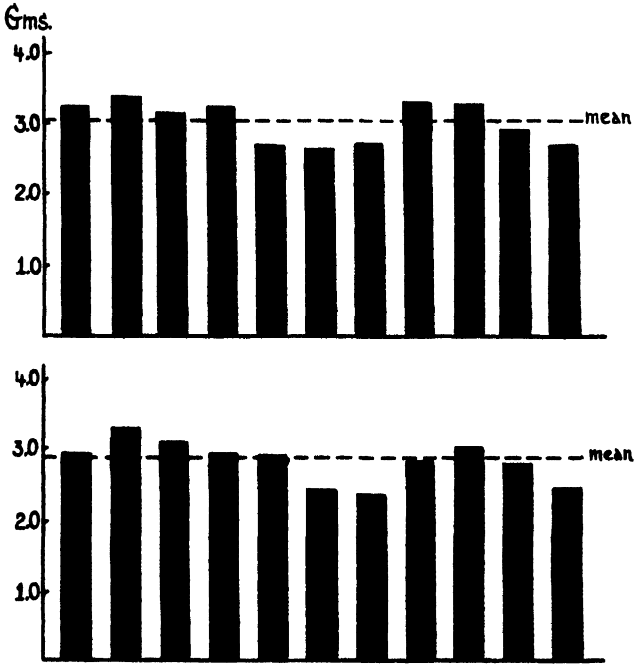

FIG. 2. Daily urinary excretion of potassium in two normal human subjects

In Table III are shown results on the recovery of potassium added to urine. The error in recovery with the silver cobaltinitrite method was generally less than 3 per cent. Table III also shows a comparison of the present method with the gravimetric chloroplatinate method described by MacKay and Butler.[2] The results obtained by the two methods with standard solutions, with urines and with known amounts of potassium added to urines, are in good agreement with each other. Usually the two methods give results which agree within 3 per cent. Results by each method are, for the most part, within 2 per cent of the theoretical values for standard solutions of potassium sulfate and for known amounts of potassium added to urine. In general, the results obtained with the chloroplatinate method are slightly lower than with the silver cobaltinitrite method.

TABLE IV

Determination of Potassium in Tissues. Chloroplatinate and Ag Cobaltinitrite Gravimetric Methods Compared

Rat tissue	Fresh weight of sample	Cobaltinitrite (10 ml aliquot)	Chloroplatinate	
		K	Size of aliquot	K
	gm	*mg. per gm*	*ml*	*mg per gm.*
Heart	1.1306	2.53	7	2.66
	1.2575	2.48	5	2.42
Liver	3.3405	2.92	5	2.88
	3.9173	3.21	5	3.24
Gastrocnemius muscle	1.1912	3.91	5	3.84
	1.0910	4.03	7	3.93

2 ml. of 10 N H_2SO_4 were added to each tissue sample in a vitreosil evaporating dish. After digesting for 2 hours on the steam bath, the samples were ashed overnight at 540° in a muffle furnace. Each ash was dissolved in 20 ml. of distilled water and filtered. Aliquots as indicated were used for the determinations of potassium.

In Table III data are also included which indicate that the concentrations of sodium, magnesium, calcium, and phosphorus which ordinarily occur in urine do not interfere with either method.

Several hundred determinations on human urines, not included in this report, have provided a basis for judging the method as suitable for routine analysis. The daily excretion levels of potassium found in two normal subjects are illustrated in Fig. 2. These subjects were maintained on a constant potassium intake, the daily diet containing, by analysis, 3.27 gm. of potassium.[3] The determinations for these two subjects show average daily excretion values for 11 successive days of 2.84 and 3.02 gm. respectively.

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[2] *Cf.* (7), p. 729.

[3] 1 day's food supply as served, including fluids, was weighed and reduced to a homogeneous mass by means of a Waring mixer. A 25 gm. aliquot of the diet was evaporated in the presence of sulfuric acid, ashed in a muffle furnace, extracted, and analyzed as described for urinary potassium.

The maximum variation from the mean was 0.46 gm. The discrepancy between intake of potassium and recovery from urine is undoubtedly due to loss of this element in feces and perspiration.

The procedure here described is more convenient and less time consuming than the gravimetric chloroplatinate method. It eliminates the necessity for evaporating aliquots of the ash extract to dryness, for extracting the precipitated chloroplatinate with aldehyde-free 80 per cent ethyl alcohol, as well as the transfer of precipitate to a filter. If one uses a 5 ml. aliquot of urine and, after evaporation, ashes over an open flame, it is possible to complete a potassium analysis within 2 hours after the urine is voided. Ashing in a muffle furnace at a low temperature is preferable as it eliminates the error produced by spattering and the possibility of a temperature sufficiently high to volatilize a significant amount of potassium.

Preliminary results (Table IV) indicate that this method is applicable to the analysis of tissues such as liver and muscle.

SUMMARY

It has been shown that, under prescribed conditions, the weight of a potassium silver cobaltinitrite precipitate is in relatively constant ratio to the amount of potassium in the solution thus precipitated. This fact has been used as the basis for a gravimetric method of estimating urinary and tissue potassium. The method gives recovery values on known solutions that are generally within 2 per cent of the theoretical values. A satisfactory agreement was found between the results obtained with the gravimetric chloroplatinate and silver cobaltinitrite methods. The daily potassium excretion was determined on two normal subjects maintained on a constant potassium intake.

BIBLIOGRAPHY

1. Burgess, L L , and Kamm, O., *J. Am Chem. Soc* , **34**, 652 (1912).
2. Breh, F., and Gaebler, O. H., *J. Biol. Chem.*, **87**, 81 (1930).
3. Truszkowski, R., and Zwemer, R. L., *Biochem. J.*, **30**, 1345 (1936).
4. Harris, J. E., *J. Biol. Chem.*, **136**, 619 (1940).
5. Weichselbaum, T. E., Somogyi, M., and Rusk, H. A., *J. Biol. Chem.*, **132**, 343 (1940).
6. Butler, A. M., and Tuthill, E., *J. Biol. Chem.*, **93**, 171 (1931).
7. Peters, J. P., and Van Slyke, D. D., Quantitative clinical chemistry, Methods, Baltimore, 726 (1932).

SODIUM, POTASSIUM, AND CHLORIDE EXCRETION OF HUMAN SUBJECTS EXPOSED TO A SIMULATED ALTITUDE OF EIGHTEEN THOUSAND FEET*

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(Received for publication, September 29, 1944)

The major electrolyte change for a man working and living at 5.34 to 5.80 km. was shown by Dill and coworkers (1) to be a decrease in bicarbonate and sodium content of the serum and some increase in its chloride content. It was demonstrated by Davies, Haldane, and Kennaway (2) and by Leathes (3) that forced breathing of air resulted in a diuresis of an alkaline urine. It was also shown (2) that the increased alkalinity of the urine was largely due to bicarbonates.

It is possible that a reduced oxygen tension in the atmosphere may directly or indirectly influence the excretion of sodium and potassium in the urine; yet there is surprisingly little information on this subject in the literature. The possibility that the requirements for these elements might be altered in aviators or others maintained at high altitudes led to the study of this question in human subjects under conditions of simulated high altitude.

Procedure

Six young adult male subjects, aged 23 to 27 years, and varying in weight from 124 to 191 pounds, were maintained on a closely supervised regimen for a period of 3 months. The diet was the same every day, providing 2750 calories and containing 3.29 gm. of sodium and 3.27 gm. of potassium according to analysis. Chloride content of the diet was calculated to be 4.93 gm. Each subject consumed an additional 5 gm. of NaCl daily in the form of seasoning at the table. Water intake was regulated and daily activity was restricted and kept reasonably constant. 24 hour urine specimens were routinely collected, with 10 cc. of 10 N sulfuric acid added as a preservative.

After a control period of 3 weeks, the subjects were exposed three times weekly to a simulated altitude of 18,000 feet. The exposures were carried out by reducing the pressure in an air-conditioned chamber. Half an hour was required to reduce the pressure in the chamber to the equivalent of 18,000 feet of altitude; this pressure was maintained for 1 hour, and another half-hour was required to restore atmospheric pressure in the chamber. The entire exposure occupied 2 hours. This procedure was continued for 8 weeks followed by a week without exposure.

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* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Department of Physiology, Northwestern University Medical School.

During the control period and for the first 6 weeks of the exposure period, the 24 hour excretion of sodium, potassium, and chloride was determined three to five times per week. In order to determine whether or not the exposure to high altitude produced any immediate effects on electrolyte excretion, a series of tests was made on 2 hour urine specimens collected before, during, and after exposure. Similar collections were made at identical times on days when no exposure occurred. The specimens were collected for the periods from 7.00 to 9.00 a.m., 9.00 to 11.00 a.m., and 11.00 a.m. to 1.00 p.m. The subjects were allowed no breakfast on the days these tests were made and luncheon was postponed until after the final collection at 1.00 p.m. On the exposure days, the 2 hour period between 9.00 and 11.00 a.m. covered the complete exposure: ascent to 18,000 feet, 1 hour at this altitude, and descent.

Analytical Methods

Sodium was determined according to the uranyl zinc acetate method of Butler and Tuthill (4). The potassium content of the urine was determined by a gravimetric procedure after precipitation as silver cobaltinitrite. The details of this method are presented elsewhere (5). Chloride determinations were made by the Volhard-Harvey thiocyanate titrimetric method (6).

Results

The average daily quantities of sodium, potassium, and chloride excreted by each subject during the control period were compared with the average daily excretions on the days when exposure occurred, and also with the average excretions on the days during the same period when there was no exposure. This comparison is shown in Table I. It is apparent from these data that exposure to 18,000 feet of altitude produced no change in the average 24 hour excretion of sodium, potassium, and chloride.

The data obtained from analysis of the 2 hour urine collections indicate some immediate effect of altitude on the excretion of electrolytes. Fig. 1 presents the findings obtained from three separate tests, each consisting of analyses made on 2 successive days; on the 1st day no exposure occurred and on the 2nd day exposure occurred between 9.00 and 11.00 a.m. The values shown on the graphs represent the average values for the six subjects. Individual values are not shown, since the behavior of all subjects was qualitatively uniform. The average quantities of electrolytes excreted and the average urine volumes during each of the three 2 hour periods on the exposure and non-exposure days are shown. In general, the excretion of sodium, potassium, and chloride was increased during the the second 2 hour period, *i.e.*, from 9.00 to 11.00 a.m., whether or not exposure to reduced pressure occurred at this time. The increase in excretion of electrolytes was greater, however, when exposure occurred.

During the third period (11.00 a.m. to 1.00 p.m.) excretion of sodium, potassium, or chloride on the non-exposure days was never below the excretion level during the first period (7.00 to 9.00 a.m.), but was usually higher. On the exposure days the excretion during the post exposure period (11.00 a.m. to 1.00 p.m.) was generally lower than during the preexposure period or returned to the same level.

TABLE I

Average 24 Hour Excretion of Sodium, Potassium, and Chloride in Six Subjects

Subject No		Control period	Experimental period	
			Exposure days	Non-exposure days
		gm	*gm*	*gm*
I	Na	4.53	4.59	4.63
	K	2.53	2.27	2.92
	Cl	7.50	7.46	7.93
II	Na	4.42	4.88	4.92
	K	2.53	2.44	2.82
	Cl	6.89	7.70	7.98
III	Na	4.28	4.30	3.81
	K	2.68	2.34	2.52
	Cl	7.02	6.77	6.38
IV	Na	4.64	4.71	4.86
	K	2.73	2.63	3.09
	Cl	7.36	7.62	8.13
V	Na	4.98	4.57	4.69
	K	3.04	2.76	2.86
	Cl	7.83	7.26	7.55
VI	Na	4.84	5.16	5.38
	K	2.93	2.54	2.77
	Cl	7.61	8.10	8.76

In all three tests the quantity of electrolytes excreted during the time of exposure (9.00 to 11.00 a.m.) was greater than the quantity excreted in any of the 2 hour periods on the non-exposure days. In Table II the total amounts of the electrolytes excreted during the entire 6 hour period are shown for the exposure and non-exposure days. In spite of the greater excretion during the second 2 hour period when exposure occurred, the

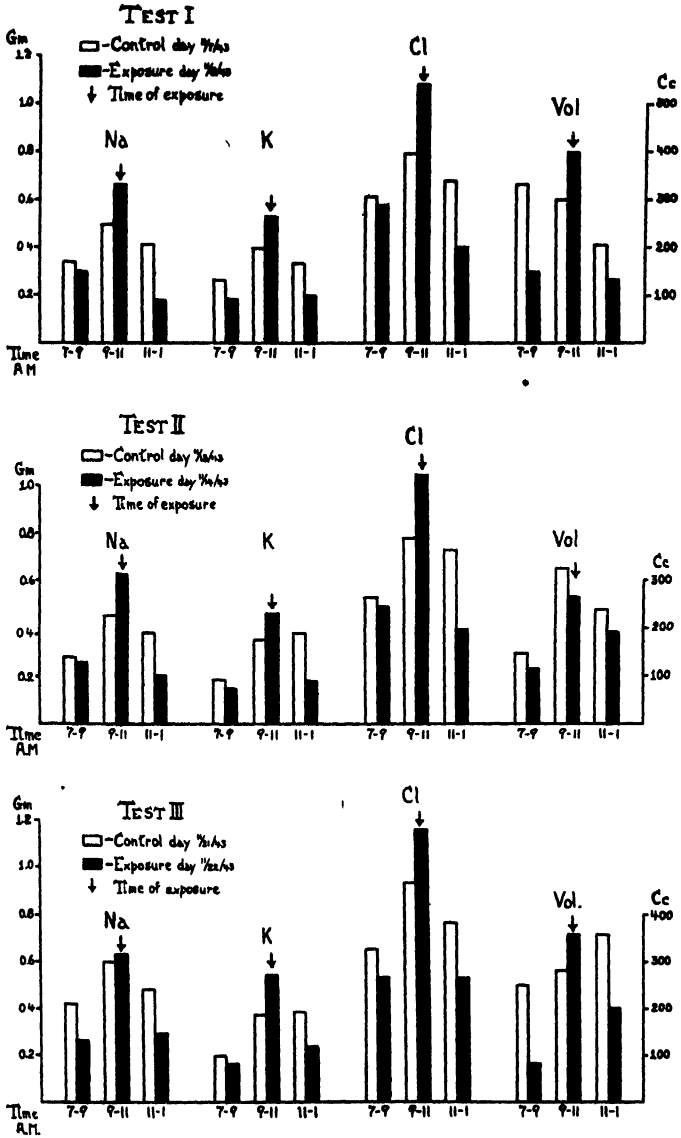

FIG. 1. Daily urinary excretion of potassium in two normal human subjects. Results of three separate tests showing average excretion of electrolytes and water by six subjects during 2 hour periods on 2 successive days. On the 2nd day of each test (black bars) the subjects were exposed to a reduced pressure equivalent to 18,000 feet of altitude for 1 hour (indicated by the arrow).

totals for the 2 days are similar, indicating that the greater reduction in excretion during the post exposure period compensated for the greater increase during the actual exposure.

During the first 2 hour period, from 7.00 to 9.00 a.m., the excretion of electrolytes was slightly lower on the exposure days. The possibility that apprehension concerning the coming exposure may have been responsible for this effect is the only explanation which presents itself.

The urine volume on the non-exposure days showed no consistent changes during the three 2 hour periods. On the days when exposure occurred, the volume was consistently greater during the actual time of exposure (9.00 to 11.00 a m.). As with the electrolyte excretion, the urine volume during the 7.00 to 9.00 a m. period was consistently lower on exposure days than on non-exposure days.

TABLE II

Average Total Excretion during 6 Hour Period from 7.00 A.m. to 1.00 P.m.

Test No		Non-exposure day	Exposure day
		gm	*gm*
I	Na	1 216	1.101
	K	0.975	0.820
	Cl	1 857	2.022
II	Na	1 073	1.049
	K	0.874	0.780
	Cl	1.968	1.886
III	Na	1.436	1.149
	K	0.907	0.919
	Cl	2.249	2.150

DISCUSSION

From the results of the 6 hour tests (7.00 a.m. to 1.00 p.m.) it was found that in our subjects the maximal excretion of electrolytes occurred between 9.00 and 11.00 a m. Leathes (3) reported an increase in alkali excretion during the morning hours and Norn (7) found that the peak of sodium, potassium, and chloride excretion occurred 3 to 6 hours after rising in the morning. Norn also established the fact that the increased excretion was definitely correlated with increased bodily activity.

The 9.00 to 11.00 a.m. period increase in mineral excretion in our subjects was augmented by maintaining the subjects at a reduced pressure equivalent to 18,000 feet of altitude during a portion of the morning. Bryan and Richetts (8) found essentially no effect of altitude on electrolyte excretion except for a slight increase in potassium excretion in two of four subjects. In our subjects the urine volume also showed an increase during the time of exposure. The increased elimination of water might be regarded as the cause of the increased excretion of electrolytes, *i.e.*, a washing out phenomenon. However, since the urine volume on the non-exposure days showed no consistent relation to electrolyte excretion, the increased elimination of water cannot be considered the sole explanation for the increase in electrolyte excretion during the exposure to reduced pressure.

The increased excretion of electrolytes cannot be explained as due to altered adrenal function inasmuch as sodium and potassium behaved similarly. The observed increase may be the result of altered metabolism or cell permeability secondary to tissue anoxia or the result of an excessive loss of carbon dioxide secondary to hyperventilation.

The reduced excretion following exposure may be regarded as a compensatory mechanism acting to restore a balanced state with respect to the electrolyte content of the body. This view is supported by the fact that the 24 hour excretion values were unchanged by exposure to reduced pressure. Whatever adaptive mechanism is involved in the maintenance of electrolyte balance apparently was unaltered by the conditions of the experiment: the degree of anoxia resulting from exposure to a pressure equivalent to 18,000 feet of altitude, the length of time each exposure lasted (1 hour), the frequency of exposure (three times weekly), and the period of time during which the exposures were repeated (8 weeks).

SUMMARY

Exposure to reduced pressure, under the conditions of the experiment, caused a temporary rise in the excretion of sodium, potassium, and chloride. Following exposure the excretion of these electrolytes was compensatorily reduced so that total excretion during the 24 hours was not altered. A temporary rise in urine volume was also found to accompany exposure.

BIBLIOGRAPHY

1. Dill, D. B , Talbott, J H., and Consolazio, W V , *J. Biol Chem.*, **118**, 649 (1937).
2. Davies, H. W., Haldane, J B. S , and Kennaway, E L , *J Physiol.*, **54**, 32 (1920–21).
3. Leathes, J. B., *Brit Med J* , **2**, 165 (1919).
4. Butler, A. M., and Tuthill, E , *J. Biol Chem.*, **93**, 171 (1931).
5. Freeman, S., and Burrill, M W., *J. Biol. Chem.*, **157**, 287 (1945).
6. Harvey, S C , *Arch. Int. Med.*, **6**, 12 (1910).
7. Norn, M., *Skand. Arch. Physiol.*, **55**, 184 (1929).
8 Bryan, A. H., and Richetts, H T., Abstracts, 27th annual meeting of the Association for Study of Internal Secretions, Chicago (1944).

THE TITRIMETRIC DETERMINATION OF "LACTOBACILLUS CASEI FACTOR" AND "FOLIC ACID"*

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Microbiological tests have aided greatly in the isolation work on "*Lactobacillus casei* factor," demonstrated by Snell and Peterson in 1940 (1), and "folic acid," the nutrient required by *Streptococcus lactis* R as defined by Mitchell *et al.* (2). It seems likely that bacterial assays will continue to play an important rôle in the study of these compounds, since development of chemical methods has been hampered by insufficient pure material, and animal tests are rather cumbersome and are complicated by intestinal synthesis.

Assays in which *Streptococcus lactis* R and *Lactobacillus casei* are employed have been described by Snell and Peterson (1), Mitchell and Snell (3), Landy and Dicken (4), and Luckey *et al.* (5). The *Streptococcus lactis* R turbidimetric method of Luckey *et al.*, has been found to produce reliable results, and the medium has been modified only to allow more acid production. In the case of *Lactobacillus casei*, reliable assays could not be obtained with media that have been published heretofore. This report presents modifications which have rendered the assay satisfactory in our hands.

The effect of the method of preparing samples on their activity for the microorganisms is not discussed, nor is there any comparison of bacterial activity with animal tests. It is apparent that the present studies on fundamental aspects of the microbiological assays are necessary to facilitate the solution of these larger problems.

EXPERIMENTAL

It was decided to use titrimetric assays, because colored or turbid samples do not affect titrations seriously. Furthermore, titrations require simpler equipment and are considered more convenient than turbidity measurements by some workers. With this method, "drifts" (marked variations in values obtained according to the portion of the curve used) are also more likely to show up.

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* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

We are indebted to Parke, Davis and Company, Detroit, Michigan, for the crystalline vitamin B_c used in these studies and to Mrs. William Pollard for testing the modified media in the analysis of foods.

The general procedure conforms to that outlined by Luckey *et al.* (5) with the following exceptions. The inoculum medium for *Streptococcus lactis* R contains 1.0 mg. of solubilized liver extract per 10 cc. of medium and the *Lactobacillus casei* inoculum medium contains 0.1 mg. of solubilized liver extract per 10 cc. of medium. Vitamin B_c (6) is used as a standard, because different batches of solubilized liver extract have been found to vary in potency. Solutions of vitamin B_c are quite stable when refrigerated under toluene. The assay tubes are plugged with cotton, because a longer incubation period is used. The findings of Sherwood and Singer (7) show that a refined grade of cotton must be used to prevent leaching of significant amounts of folic acid from the cotton plugs. *L. casei* assays are titrated with brom-thymol blue as an indicator, while thymol blue is a more satisfactory indicator in the titration of *S. lactis* R assays. An incubation period of 30 to 72 hours gives satisfactory results. The same medium, except for the buffers, may be used for both organisms. However, the asparagine, alanine, *p*-aminobenzoic acid, peptone, and Salts B may be omitted from the *S. lactis* R medium if desired.

Several media that have been proposed for *Streptococcus lactis* R and *Lactobacillus casei* are recorded in Table I.

Streptococcus lactis R Assay—In the case of *Streptococcus lactis* R, the problem simply involved finding the most suitable buffer, since the growth and acid production of *Streptococcus lactis* R are retarded when the pH falls below 5.2 Fig. 1 shows the acid production allowed by various buffers. The curves represent actual acid production, as blank titrations were made at each level of buffer and were subtracted from the titration value for the corresponding inoculated tubes. High concentrations of K_2HPO_4 cause excessive caramelization during autoclaving. Sodium citrate does not have this characteristic but high levels of this compound cause a considerable percentage of plugs to blow out of the tubes during the sterilization procedure. A satisfactory combination is 2.5 per cent sodium citrate plus 0.25 per cent K_2HPO_4, which allows the production of acid equivalent to about 10 cc. of 0.1 N acid per 10 cc. of medium. This is adequate for routine assays.

Table II shows the pH values of three different media which contain various concentrations of lactic acid. It is apparent that our medium is most effective in preventing a rapid drop in pH.

Fig. 2 shows a typical acid production curve obtained with *Streptococcus lactis* R, with vitamin B_c as a standard.

Lactobacillus casei Assay—When attempts were made to use previously described media for titrimetric assays for *Lactobacillus casei* factor, results were unsatisfactory for several reasons. There was not consistently good agreement between duplicate titrations at the same level of added standard

TABLE I

Media Proposed for Assay of "Folic Acid" or "Lactobacillus casei Factor"

Constituent	Amounts per 10 cc final medium				
	Snell and Peterson, 1940 (1)	Mitchell and Snell, 1941 (3)	Landy and Dicken, 1942 (4)	Luckey *et al*, 1944 (5)	Present medium
	mg	*mg*	*mg*	*mg*	*mg*
Na acetate..	60	60	60	20	200 (For *L. casei* only)
K_2HPO_4....				50	25
Na citrate.					250 (For *S. lactis* only)
Casein (acid-hydrolyzed)	50	50	50	50	50
Glucose	100	100	100	100	200
Cystine	1	1	1	1	2
Tryptophane	1	0.5	1	3	2
Adenine		0.1	0.05	0.1	0.1
Guanine		0.1	0.05	0.1	0.1
Uracil		0.1	0.05		0.1
Xanthine		0.1	0.05	0.1	0.1
Asparagine			2.5		1.0
Peptone treated with **norit***..					See text
dl-Alanine....					2.0
	γ	γ	γ	γ	γ
Thiamine .		1	1	2	2
Riboflavin. .	1	2	2	2	2
Nicotinic acid	2	1	2	6	6
Pyridoxine		1	4	12	12
Ca pantothenate .	5	1	2	4	4
Biotin		0.002	0.05	0.004	0.004
p-Aminobenzoic acid .					0.1
		cc	*cc*	*cc.*	*cc.*
Salts A†...	0.05	0.025	0.05		
" B‡.	0.05	0.025	0.05	0.05 (For *L casei*)	0.05

The Snell and Peterson and the Landy and Dicken media were developed for *Lactobacillus casei* The media of Mitchell and Snell and of Luckey *et al.* were developed primarily for *Streptococcus lactis* R but have also been used for *Lactobacillus casei*.

* Prepared according to Dr. George Kohler, American Butter Company, Kansas City, Kansas. 50 gm. of peptone in 200 cc. of H_2O are adjusted to pH 3 and stirred with 5 gm. of norit for 1 hour, filtered, and diluted to 500 cc.

† Salts A = K_2HPO_4 5 gm., KH_2PO_4 5 gm., H_2O 50 cc.

‡ Salts B = $MgSO_4 \cdot 7H_2O$ 10 gm., NaCl 0.5 gm., $FeSO_4 \cdot 7H_2O$ 0.5 gm., $MnSO_4 \cdot 2H_2O$ 0.337 gm., H_2O 250 cc.

and the general appearance of most of the curves obtained was not such as to inspire confidence in results obtained through their use. Most important of all, in the assay of whole liver substance with solubilized liver extract as a standard, a gradual drift from 170 to 85 per cent potency was observed as calculations were made from higher portions of the curve.

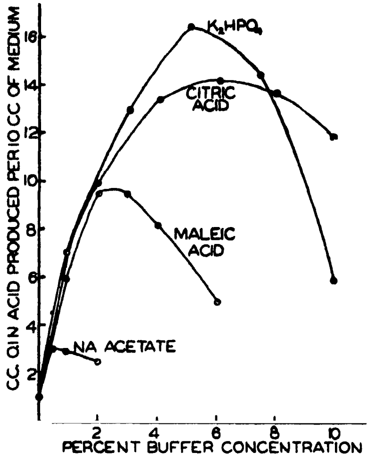

FIG. 1. The effect of buffers on acid production by *Streptococcus lactis* R. In all cases the medium was adjusted to pH 6.8 with NaOH or HCl. 2 mg. of grass juice powder, 2 mg. of whole liver substance, and 10 mg. of K_2HPO_4 were added to each tube.

TABLE II

pH Values of Streptococcus lactis R Media Containing Various Concentrations of Lactic Acid

0.1 N lactic acid per 10 cc medium	Media		
	Mitchell-Snell	Luckey *et al.*	Present medium
cc.			
0	6.9	6.9	6.9
1	5.8	6.3	6.7
2	5.3	5.9	6.5
4	4.9	5.1	6.2
6	4.6	4.6	5.8
8	4.4	4.3	5.5
10	4.2	4.2	5.2
12	4.1	4.1	5.0
15	3.9	4.0	4.7
20	3.7	3.8	4.5

The addition of alanine, *p*-aminobenzoic acid, and a peptone preparation treated with norit has eliminated these difficulties. Alanine was added because of the stimulatory action reported by Snell (8), while *p*-aminobenzoic acid was added merely as a protective measure. The peptone treated with norit is a critical factor. It should be tested to determine its effect on the blank titration and the maximum amount which does not cause an excessively high blank is then included in the medium. This amount usually lies between 1 and 3 mg. per tube. Since the upper limit of the curve is quite high, we do not consider a blank of 2 to 3 cc. excessive. Fig. 3 shows a typical acid production curve obtained with *Lactobacillus casei*, with vitamin B_c as a standard.

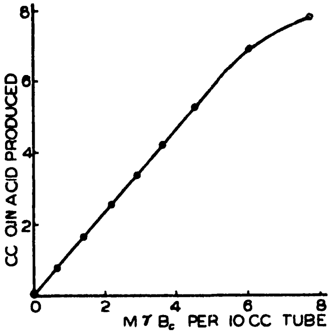

FIG. 2. Acid production of *Streptococcus lactis* R in response to graded amounts of vitamin B_c.

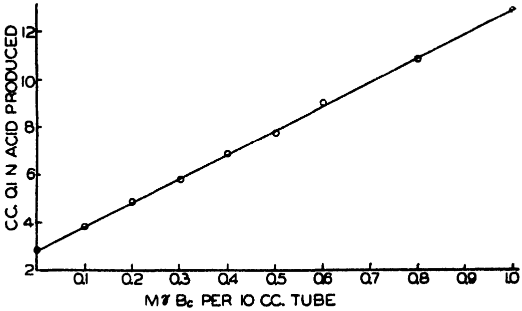

FIG. 3. Acid production of *Lactobacillus casei* in response to graded amounts of vitamin B_c.

In "side by side" assays in which vitamin B_c was compared with solubilized liver extract, wide ranges of potency were found with curves obtained with three different media. These are tabulated in Table III. In the case of the media of Mitchell and Snell and of Luckey *et al.*, duplicate titrations at the same level of added supplement agreed fairly well, but values obtained from different levels of added standard showed a pronounced drift. This difficulty has not been encountered with our medium and, furthermore, whole liver has been assayed with vitamin B_c as a standard without interference from drifts.

Although our medium has been successfully employed in the analysis of a large number of natural materials, it is not unlikely that certain samples may have to be subjected to ether extraction or some other procedure to prevent drifts.

TABLE III

Vitamin B_c Content of Solubilized Liver Extract As Obtained with Lactobacillus casei and Three Different Media

Medium	Range of values obtained from various portions of curves
	γ per gm
Mitchell-Snell ..	6.2- 9.4
Luckey *et al.*	9.3-43
Present medium...	22 -24

TABLE IV

Vitamin B_c Content of Miscellaneous Materials

The liver powders were assayed directly and the other samples were treated with taka-diastase.

	Streptococcus lactis R	*Lactobacillus casei*
	γ per gm	*γ per gm*
Solubilized Liver Extract I.	11.4	25
" " " II.. ...	8.4	23
Whole liver substance. ..	13.5	20
1:20 liver powder .	15	7.3
Beef liver, fresh	0.97	0.80
Rat " "	0.81	0.82
Linseed oil meal	0.43	0.45

When the blank is low, a sigmoidal curve is occasionally obtained with *Lactobacillus casei*. This may be eliminated by setting the basal medium at pH 6.0 to 6.5 rather than pH 6.8 to 7.0. When enough peptone preparation is added to bring the blank titration to 2 cc. or more, sigmoidal curves are not obtained.

The vitamin B_c content of several materials is reported in Table IV. The authors feel that the expression of "folic acid" or "*Lactobacillus casei* factor" potency should be in terms of some crystalline compound. The variation in potency of solubilized liver extracts as measured by *Strepto-*

coccus lactis R emphasizes this need. In the present studies, vitamin B_c has been used because of its availability and because very small amounts are needed to produce a response by either organism. For the latter reason, it is probably a more convenient standard than some of the other "folic acid" compounds that have been isolated (9).

DISCUSSION

Collaborative studies within our laboratory and with other workers have convinced us that, with any particular medium, essentially the same values are obtained with either turbidity or titration measurements, provided the sample itself does not interfere with turbidity readings. However, with respect to the detection of drifts, turbidity measurement as ordinarily employed does not subject a medium to as severe a test as measurement of acid production.

It would of course be far preferable to use pure materials instead of the peptone preparation in the basal medium. However, the present studies are being reported because it may take considerable time to determine what the stimulatory substances are.

The modifications reported in this paper have been employed in our laboratory for the routine analysis of a number of foods with consistently good results.

SUMMARY

1. The *Streptococcus lactis* R medium of Luckey *et al.* (5) has been modified to allow more acid production and thus make it more suitable for titrimetric assays for "folic acid."

2. The use of previously described media for "*Lactobacillus casei*" assay of "*Lactobacillus casei* factor" gave unsatisfactory results. The difficulties have been eliminated by providing additional supplements in the basal medium.

BIBLIOGRAPHY

1. Snell, E. E., and Peterson, W. H., *J. Bact.*, **39**, 273 (1940).
2. Mitchell, H. K., Snell, E. E., and Williams, R. J., *J. Am. Chem. Soc.*, **63**, 2284 (1941).
3. Mitchell, H. K., and Snell, E. E., *Univ. Texas Pub., No. 4137*, 36 (1941).
4. Landy, M., and Dicken, D. M., *J. Lab. and Clin. Med.*, **27**, 1086 (1942).
5. Luckey, T. D., Briggs, G. M , Jr., and Elvehjem, C. A., *J. Biol. Chem* , **152**, 157 (1944).
6. Pfiffner, J. J., Binkley, S. B., Bloom, E. S., Brown, R. A., Bird, O. D., Emmett, A. D., Hogan, A. G , and O'Dell, B. L., *Science*, **97**, 404 (1943).
7. Sherwood, M. B., and Singer, E. D , *J. Biol. Chem.*, **155**, 361 (1944).
8. Snell, E. E., *Proc. Soc Exp. Biol. and Med.*, **55**, 36 (1944).
9. Luckey, T. D., Teply, L. J., and Elvehjem, C. A., *Science*, **100**, 201 (1944).

THE EFFECT OF DIETARY FAT ON THE LIPOTROPIC ACTION OF INOSITOL

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The possibility that choline exerts lipotropic action through its introduction into newly formed lecithin molecules is an attractive hypothesis for which there exists some supporting evidence (1–3). Inositol, which is also a constituent of phospholipid (4, 5), may possibly exert its effect in the same way. McHenry and Patterson (6) have already suggested this possibility. If these two substances actually do lower liver fat by virtue of their incorporation into phospholipid molecules, then essential fatty acids, which also constitute integral parts of certain phospholipids, might similarly be required before choline and inositol could exert their effects, or be necessary to produce maximal lipotropic action.

About the time that experimental work was started to test these points, Engel published a paper (7) in which he mentioned the relation of the essential fatty acids to the lipotropic action of choline. His motives for carrying out this particular investigation differed from those which initiated the present study. Engel had found that pyridoxine is necessary for choline to exert its full lipotropic effect, and because of the complementary relationship of pyridoxine and essential fatty acids in the cure of rat acrodynia (8, 9) he was prompted to examine the possible influence of essential fatty acids on the lipotropic action of choline. Engel fed 0.1 ml. of corn oil to each test rat daily. This supplement did not alter the lipotropic effect of choline in short term experiments (3 weeks), but did augment the latter's action in an experiment of 8 weeks duration. In a recent publication (10) by one of us, data obtained from a small series of animals supported this finding of Engel's. Unexpectedly, it was also discovered that corn oil (Mazola brand), which was fed with the object of supplying essential fatty acids, instead of augmenting the lipotropic action of inositol actually obliterated it. The present paper confirms this observation and reports more detailed analytical data for liver fat from rats on a number of diets similar to those utilized in the preliminary work reported by Beveridge (10).

EXPERIMENTAL

Young rats of the Wistar strain were used for the feeding experiments which ran 8 weeks. The fat-free basal diet consisted of the following components:[1] casein (fat- and vitamin-free, Smaco brand) 8 per cent, gelatin 12 per cent, sucrose 73 per cent, salt mixture[2] 5 per cent, agar 2 per cent, cod liver oil concentrate (500,000 I.U. of vitamin A per gm. and 50,000 I.U. of vitamin D per gm. (Ayerst, McKenna and Harrison)) 0.015 per cent. The casein and gelatin were extracted with hot alcohol-ether.

The B vitamins were administered daily by subcutaneous injection in 0.5 ml. of 0.9 per cent saline: thiamine hydrochloride 50 γ, riboflavin 25 γ, pyridoxine 20 γ, Ca pantothenate 100 γ, nicotinic acid 100 γ. The supplements, which were added at the expense of the sucrose, were given as described in the text.

Series A—This series was planned to test the effects of essential fatty acids (supplied by feeding corn oil) on the lipotropic action of choline and inositol. Twenty young rats (23 to 35 days old) were used for each group and litters were divided as evenly as possible among the different groups. The groups were also balanced with respect to weight and sex.

The diets were made up fresh every 10 to 14 days. The fatty ingredients were incorporated by spraying them, in dilute solution in acetone, over thin layers of the other components, which were then thoroughly mixed with a spatula until all odor of the solvent had disappeared. Further mixing was done by crumbling the mass by hand. The diets were stored at 4° in tightly closed containers. The animals were fed *ad libitum*.

With the exception of Groups 6B and 7B individual liver fat was determined in the usual way by saponification, acidification, and extraction of the fatty acids[3] with petroleum ether. The livers of Groups 6B and 7B were pooled and the fat was extracted essentially according to the methods described by Artom and Fishman (12). Iodine numbers were determined according to the method of Rosenmund and Kuhnhenn as modified by Yasuda (13). Phospholipid was estimated by multiplying the total phosphorus of the lipid fraction by the Artom and Fishman (12) factor 22.7; cholesterol was determined gravimetrically as the digitonide. Details of the supplements and the analytical findings are given in Tables I and II.

Series B—Since the results of Series A had shown that, under the conditions used, the inclusion of corn oil obliterated the lipotropic action of inositol, the effect of a saturated fat (free of essential fatty acids) was also determined. At the same time the effect of this fat was compared with that of corn oil on the lipotropic action of choline. In order to avoid any complicating factors due to differences in food intake, all the groups were

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[1] The composition of the basal diet, as described in the preliminary report (10), is confused by a typographical error The words "12 per cent casein" should be deleted

[2] The salt mixture used was one based on the McCollum Salt Mixture 185 (11) modified according to the best available data concerning the mineral requirements for growing rats The composition, in gm per 100 gm. of salts, is as follows Ca lactate ($5H_2O$) 35 15, $CaCO_3$ 5 28, $Ca(H_2PO_4)_2 \cdot H_2O$ 14.60, K_2HPO_4 6 45, $NaH_2PO_4 \cdot H_2O$ 18.76, NaCl 9.34, $MgSO_4$(anhydrous) 7 19, ferric citrate ($3H_2O$) 3.19, $MnSO_4$ $2H_2O$ 0.33, $ZnSO_4 \cdot 7H_2O$ 0.035, $CuSO_4 \cdot 5H_2O$ 0 039, KI 0 00039.

[3] The material extracted, which is usually referred to as "crude liver fatty acids," contains also the unsaponifiable matter.

TABLE I

Effect of Corn Oil on Lipotropic Action of Choline and Inositol, Series A

Groups of twenty rats, average weight 62 gm., were fed experimental diet for 8 weeks.

Group No	Supplement	No. of survivors	Change in weight of survivors	Food intake	Fatty acids as per cent of wet liver weight Average
	per cent		*per cent*	*gm.*	
1	None (basal)	14	+24	7.8	23.4
2	Choline chloride (0.5)	16	+71	9.9	6.4
3	Inositol (0.3)	13	+20	8.6	13.3
4	Corn oil (1.0)	15	+25	7.9	25.3
5	" " (1.0) + inositol (0.3)	11	+33	8.7	27.2
6A	" " (1.0) + choline chloride (0.5)	17	+112	9.6	4.7
7A	" " (1.0) + choline chloride (0.5) + inositol (0.3)	19	+105	9.8	3.8
6B	Same as Group 6A	17	+94	8.8	4.5*
7B	" " " 7A	16	+99	9.7	3.6*

* Total lipids extracted from the pooled livers by alcohol and alcohol-ether gave values for Group 6B of 6.3 and for Group 7B of 4.6 per cent wet liver weight. Aliquots of these extracts were used for determination of total fatty acids in the usual way by saponification, acidification, and extraction with petroleum ether.

TABLE II

Composition of Fats from Pooled Livers, Series A

The values are expressed as per cent of fat-free, dry liver tissue. The per cent liver residue weight of original wet weight was 22 15 in Group 6B and 23.95 in Group 7B.

Group No	Total lipid*	Phospholipid†	Cholesterol		Steryl ester‡ calculated as oleate	Glyceride§
			Free	Esterified		
6B	24.8	13.30	0.66	0.37	0.62	10.2
7B	19.2	11.90	0.63	0.37	0.62	6.0

* The iodine number of Group 6B was 81.3 and of Group 7B was 92.1.

† Calculated from P in total lipid (chloroform-soluble P × 22.7).

‡ Esterified cholesterol × 1.68 assumed to give the weight of cholesteryl esters.

§ By difference.

paired fed on Group 4 ("non-essential fat" supplement). The supplements used in this experiment and their effects upon the rats are shown in Table III and the proximate liver analyses are given in Table IV.

The livers, after being weighed and pooled, were emulsified in a Waring blendor with 5 volumes of acetone. Small aliquots were taken for total crude fatty acids, which were determined in the usual way. The remainder of the acetone suspension was made up to about 10 volumes and allowed to stand for about half an hour. The acetone dehydrated the liver tissue and took up a portion of the liver fat. The mixture was centrifuged, the supernatant was filtered, and the filtrate was evaporated to dryness *in vacuo* under a stream of nitrogen. After dehydration of the material left in the flask, by addition of absolute alcohol and evaporation *in vacuo*, the fat in the residue was extracted with petroleum ether. The dehydrated and partially defatted liver tissue was extracted in Pyrex centrifuge pots (250 ml.) five times by refluxing for about 30 minutes each time with 3

TABLE III

Effect upon Young Rats of Diets Used, Series B

Groups of twenty rats were fed experimental diets for 8 weeks; the food intake was 7.9 gm. per rat per day.

Group No	Supplements	Average initial weight	Survivors	Average initial weight of survivors	Average change in weight of survivors	Average wet liver weight
	per cent	*gm*		*gm*	*per cent*	*gm*
	None (basal)	68.8	10	75.1	+8	5.10
1	Corn oil (1.0)	70.4	16	77.8	+31	7.52
2	Inositol (0.3)	70.2	13	82.5	+10	5.15
3	Corn oil (1.0) + inositol (0.3)	69.9	15	78.5	+20	7.15
4	Non-essential fat* (1.0)	69.3	12	79.2	+16	6.10
5	" " (1.0) + inositol (0.3)	72.5	13	72.9	+27	4.46
6	Corn oil (1.0) + choline chloride (0.5)	72.4	20	72.4	+36	3.56
7	Non-essential fat (1.0) + choline chloride (0.5)	69.1	18	65.1	+41	3.23

* This material, isolated from beef dripping by repeated fractional crystallization from acetone, had an iodine number of only 5.0. Even if all the unsaturation were due to linoleic acid, the quantity supplied in the diet would amount to only about 2 mg per rat per day. However, this possibility is most unlikely. In the first place, published analyses of beef dripping show that linoleic acid accounts for only a comparatively small part of the unsaturation of this fat (oleic acid 40 to 50 per cent, linoleic acid 2 to 3 per cent of the fat). Further, since the more unsaturated fats would be preferentially left behind in the fractionation, the small amount of unsaturated glycerides remaining in the fraction is almost certainly due to oleic acid. The melting point of the isolated fraction, which will be referred to as "non-essential fat," was 50-53°.

volumes of absolute alcohol. The alcoholic extracts were evaporated as above and the petroleum ether extract of the residue so obtained was combined with that from the acetone extract. The water-free, fat-free liver tissue was dried *in vacuo* over P_2O_5 and weighed. Saponification of this liver residue revealed that the process used for extraction of the fat was more than 99.5 per cent efficient. Cholesterol was determined by the Schoenheimer-Sperry method (14). Phospholipid was estimated by (*a*) a calculation based on the total phosphorus in the petroleum ether extract by using the factor 25.2 (assuming 93 per cent monoamino phosphatide and 7 per cent diamino phosphatide); (*b*) a calculation based on the phosphorus content of the total crude phospholipid precipitated by acetone and $MgCl_2$.

TABLE IV

Effect of Dietary Fat on Lipotropic Action of Choline and Inositol, Series B

Groups of twenty rats, average weight 70 gm., were fed experimental diets for 8 weeks; the food intake was 7.9 gm per rat per day.

Group No	Fatty acids		Total lipid		Unextracted fat	Water	Dry tissue
	per cent wet liver	*per cent dry, fat-free liver*	*per cent wet liver*	*per cent dry, fat-free liver*	*per cent of fat extracted*	*per cent wet liver*	*per cent wet liver*
	30.4	200	31.2	206	0.12	53.6	15.2
1	31.0	241	32.4	253	0.14	54.7	12.9
2	20.0	132	23.5	157	0.26	61.3	15.0
3	28.0	191	33.8	231	0.09	51.5	14.7
4	25.7	173	28.4	191	0.06	56.7	14.9
5	21.0	119	25.1	143	0.11	57.3	17.6
6	5.6	24	8.0	35	0.47	68.7	23.3
7	4.3	19	7.1	31	0.29	69.7	23.2

This precipitation was carried out in 250 ml. centrifuge pots by taking about 50 mg. of liver fat, dissolving this in 5 ml. of petroleum ether, and adding 85 ml. of anhydrous acetone. According to the recommendation of Sinclair and Dolan (15), the precipitation was completed by adding 0.25 ml. of a saturated ethanolic solution of magnesium chloride. After being thoroughly mixed, the material was left in the refrigerator for about an hour. The mixture was centrifuged and the precipitate was washed with 25 ml., 15 ml., and 10 ml., respectively, of acetone. Phosphorus in the precipitate was determined by the method of King (16).

Iodine numbers on the unfractionated fats were determined in the usual way (13).

DISCUSSION

There has been no unanimity among investigators working on lipotropic substances as to the best method of expressing analytical data derived from lipid determinations on livers of markedly variable fat content. Results have been most commonly expressed as a per cent of the wet weight of liver. The variability of water content of the liver with age and with the degree of infiltration of fat may not only conceal changes which have occurred in the absolute amounts of the lipids present but may also lead to quite erroneous conclusions. Beeston, Channon, and Wilkinson (17) have advocated expressing results as gm. of lipid per 100 gm. rat; *i.e.*, liver fat as a percentage of final body weight. Since the addition or withdrawal of one nutrient often may have a marked effect on body weight without a corresponding effect on the liver, the objection to this method of expressing data is obvious. The use of the initial body weight, while not ideal, avoids some of the difficulties. Some investigators (*e.g.*, Artom and Fishman (12)) have based their results on the *fat-free* tissue and others (*e.g.*, Engel (7)) on the *moisture-free* tissue. While the two latter methods constitute an improvement, they still leave something to be desired, since in each case insufficient cognizance is taken of one large variable. The present authors believe that liver fat should be expressed as a percentage of the weight of the water-free, fat-free liver since the latter, in the absence of extensive cirrhosis, is a fairly true measure of the relative amounts of active tissue. The data obtained in the present study are therefore so expressed, unless otherwise stated.

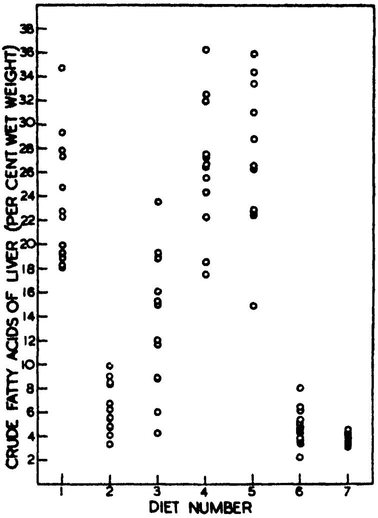

FIG. 1. Individual liver fat values, obtained on diets in Series A

The distribution of the individual values for total crude liver fatty acids of Series A is shown in Fig. 1 and the summarized data are presented in

Table I. The data in Table I reveal a curious phenomenon for which no explanation can yet be advanced. On a fat-free diet the lipotropic effect of inositol was definitely demonstrated (Group 3) but the addition of corn oil (Mazola) to the diet completely prevented this action (Group 5). The observation was confirmed in Series B (Table IV (compare Groups 2 and 3)). The addition of an identical amount of a solid fat (iodine No. 5), which may be assumed for all practical purposes to be devoid of essential fatty acids, had no such nullifying effect upon the lipotropic action of inositol (Table IV (compare Groups 2 and 5)). Obviously some material in the Mazola modifies the action of inositol. Since glycerides of the essential fatty acids are major components of corn oil, the possibility that these substances are the agents responsible for the effect is a likely one, although this point has not yet been definitely established.

Engel's report (7) that corn oil augments the lipotropic action of choline appears to be supported by the data in Table I (compare Group 2 with Groups 6A and 6B). Differences in the average values are not of large magnitude but the fact that fifteen out of seventeen individual values in Group 6A are below the average value for Group 2 (see Fig. 1), coupled with the good agreement between the results in Groups 6A and 6B, is evidence that the differences are significant. The data in Table I (compare Group 6A with Group 7A and Group 6B with Group 7B) also appear to confirm Engel's finding that inositol brings about a further lowering of liver fat when added to diets containing an adequate amount of choline, for all nineteen individual values in Group 7A are below the average value for Group 6A. Unequivocal proof of the reality of the supplementary lipotropic effects of corn oil and inositol in diets containing adequate choline would require much larger groups of animals than were utilized in the present studies. However, such effects as were observed at the high levels of choline used in these experiments, although small, were in the direction expected from Engel's work.

Fractionation of the lipids from Groups 6B and 7B of Series A revealed that the reduction of liver fat brought about by the addition of inositol to a diet containing an adequate amount of choline is due mainly to a decrease in neutral fat (Table II).

Although in Series B the percentage of phospholipid in the different fats varied widely (6.9 to 60.6 per cent, Table V), the phospholipid levels calculated on the weight of dry, fat-free liver tissue remained practically constant in the livers of the different groups (Table VI), despite the fact that a number of the diets contained neither choline nor inositol and some were deficient in essential fatty acids. The fact that the total amount of phospholipid was relatively unchanged whether choline was present or not warrants further examination of the phospholipid fractions from the animals

TABLE V

Analytical Data for Fats from Pooled Livers, Series B

The values are expressed as per cent of total lipid.

Group No	Phospholipid*†	Cholesterol		Steryl ester‡	Glyceride§	Iodine No. of total lipid
		Free	Esterified			
Basal	7.5	0.66	2.27	3.82	88.0	70.2
1	7.1	0.55	1.42	2.39	90.0	82.8
2	10.8	0.69	2.04	3.44	85.1	73.4
3	6.9	0.54	1.43	2.41	90.2	83.0
4	8.8	0.67	1.72	2.90	87.6	71.7
5	12.3	0.82	1.43	2.41	84.5	70.2
6	47.1	2.70	0.85	1.43	48.8	76.1
7	60.6	3.31	1.76	2.96	33.1	68.8

* Calculated from the phosphorus of total lipid (petroleum-soluble P × 25.2).

† The phospholipid figures were those obtained by the method referred to as (*a*) in the text; values by method (*b*) were about 13 per cent lower. However, the values by method (*a*) agreed very closely (usually within 5 per cent) with those calculated from the weight of fatty acids derived from the phospholipid precipitated by the method of Sinclair and Dolan (15). In this latter case, aliquots of the petroleum ether solutions of the total lipids were taken to contain about equal quantities of petroleum-soluble phosphorus. These were evaporated to dryness, taken up in 10 ml. of light petroleum, and treated with 175 ml. of anhydrous acetone. Precipitation was completed by adding 0.45 ml. of ethanolic magnesium chloride solution. The washed precipitates were saponified with 5 ml. of 0.4 N alcoholic KOH, acidified, and extracted with light petroleum. The weight of fatty acids × 1.48 (see Artom and Fishman (12)) was assumed to give the phospholipid in the aliquot.

‡ The esterified cholesterol × 1.68 is assumed to give the cholesteryl ester.

§ By difference.

TABLE VI

Nature and Amount of Liver Lipids, Series B

The values are expressed as per cent of fat-free, dry liver tissue.

Group No	Phospholipid (P × 25.2)	Cholesterol		Glyceride
		Free	Esters*	
Basal	15.4	1.36	7.84	181
1	18.0	1.39	6.02	228
2	16.9	1.08	5.37	134
3	15.8	1.24	5.55	209
4	16.8	1.28	5.53	167
5	17.6	1.17	3.44	121
6	16.2	0.93	0.49	16.9
7	18.7	1.02	0.90	10.2

* Calculated as oleic ester.

on the different diets to determine what changes, if any, have taken place in the choline-containing lipids.

The amount of free cholesterol remained practically constant in all the groups but marked differences in the cholesteryl esters were observed. The average values, expressed as absolute weight of esterified cholesterol per liver, were as follows: on the basal diet 36.1 mg., on Diets 1 to 7, 34.4, 24.7, 34.6, 29.5, 16.0, 2.4, and 4.0 mg., respectively. The high value found for cholesteryl esters on the basal diet is worthy of note. In view of the prominent lipotropic rôle which has been assigned to inositol in controlling cholesterol levels in the liver, it was surprising to find that on the diets used in these studies choline had a much more marked effect than inositol in reducing cholesteryl esters (Table VI, compare Groups 3 and 6). Similar observations were made 10 years ago when Best *et al.* (18) noted that, in rats fed a grain diet, addition of choline caused an appreciable decrease (33 per cent) in the glyceride content of the liver, but a much larger percentage decrease (78 per cent) in the steryl esters. When 40 per cent beef dripping was incorporated into the grain diet, Best *et al.* found that the percentage decrease in steryl esters occasioned by adding choline was even greater (87 per cent). A similar but less marked decrease (59 per cent) in steryl esters was caused by choline in grain diets containing 20 per cent of Crisco and 2 per cent of cholesterol. Inositol and other lipotropic factors were undoubtedly present but were constant in the diets under comparison.

When inositol was added to a diet containing the "non-essential fat," a definite lowering in the cholesteryl esters took place (compare Groups 4 and 5, Table VI), but when inositol was added to a diet containing corn oil and choline, no change in steryl esters was observed (Table II), although a 41 per cent decrease in glycerides was produced. The whole question of the lipotropic action of inositol and the effect of the nature of the basal diet on its action merits further study.

Corn oil plus choline gave the lowest cholesteryl esters resulting from any of the diets used in these experiments (Group 6, Tables V and VI).

The proportion of glyceride in the fats (Table V) shows very wide fluctuations. In general, the variations in the other lipid components appear to be affected to a greater or lesser degree by simple dilution with accumulating glycerides. While the principal action of choline upon the *total amount* of liver lipids as revealed by these studies is to reduce the glycerides, a fact already noted by other workers using different basal diets and different supplements, a similar (and in some cases even greater) proportional reduction in steryl esters is brought about by this lipotropic agent.

The iodine numbers (Table V) of the fats fell into the order expected of them, *i.e.*, the fats of those groups receiving corn oil possessed the higher iodine numbers.

The experiments were not designed to investigate the effect of these supplements upon growth, but it is interesting to note that the animals receiving choline grew much better and were healthier in appearance than the other rats. Best *et al.* (19) have already noted a similar effect of choline; McHenry (20) has reported more extended studies of this and has discussed the beneficial influence of choline on growth.

Scaly tail and occasionally other signs usually taken as indicative of essential fatty acid deficiency (21, 22) were sometimes observed (particularly in Series A). Some of the rats receiving corn oil showed similar scaly tails. It is worthy of note that those groups receiving choline, even in the absence of corn oil, displayed little or no sign of the disease. The inclusion of "non-essential fat" apparently aggravated the condition, for the scaliness was most marked in Group 4, Series B. The real nature of the deficiency observed in these experiments is not understood. The possibility should not be overlooked that the various supplements used may have caused alterations in the intestinal flora, with consequent changes in the B vitamins and other metabolites synthesized in the gut (23).

The mortality of those rats not receiving choline was quite high due to the development of hemorrhagic kidneys. A few of the surviving rats which had received no choline had cirrhotic livers (confirmed by examination of sections) and all of them had "frosted" or badly pitted kidneys.

A few of the rats in the basal group of Series B, which died of hemorrhagic kidneys, had also areas of hemorrhage which followed roughly the distribution of the pancreas. This phenomenon was not noticed in any of the other groups.

Before any interpretation of the findings can be attempted, it will be necessary to determine whether the nullifying influence of corn oil upon the action of inositol is due to the essential fatty acids contained therein or whether some other component is involved. The problem is under investigation in this laboratory.

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SUMMARY

1. Corn oil obliterates the lipotropic action of inositol under the dietary regimen used.

2. Choline brings about a greater reduction of cholesterol esters than does inositol under the conditions used.

3. Liver phospholipid is practically unaffected by the removal of choline, inositol, corn oil, or "non-essential fat" from the diet. The addition of these factors singly or in pairs did not change appreciably the amount of phospholipid in the liver.

BIBLIOGRAPHY

1. Welch, A. D., *Proc. Soc. Exp. Biol. and Med.*, **35**, 107 (1936).
2. Welch, A. D., *J. Biol Chem.*, **137**, 173 (1941).
3. Stetten, D , Jr , *J. Biol Chem.*, **138**, 437 (1941).
4. Anderson, R. J., and Roberts, E. G., *J. Biol. Chem.*, **89**, 599 (1930).
5. Folch, J., and Woolley, D. W., *J. Biol. Chem.*, **142**, 963 (1942).
6. McHenry, E. W., and Patterson, J. M., *Physiol. Rev.*, **24**, 128 (1944).
7. Engel, R W , *J Nutr* , **24**, 175 (1942).
8. Birch, T. W., *J. Biol. Chem.*, **124**, 775 (1938)
9. Salmon, W. D., *Proc. Am. Soc. Biol. Chem* , *J. Biol. Chem.*, **133**, p. lxxxiii (1940).
10. Beveridge, J. M. R., *Science*, **99**, 539 (1944).
11. McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, **33**, 63 (1918).
12. Artom, C., and Fishman, W. H , *J. Biol. Chem.*, **148**, 405 (1943).
13. Yasuda, M., *J. Biol. Chem.*, **94**, 401 (1931-32).
14. Sperry, W. M., and Brand, F. C., *J. Biol. Chem.*, **150**, 315 (1943).
15. Sinclair, R G., and Dolan, M., *J. Biol. Chem.*, **142**, 659 (1942).
16. King, E J., *Biochem. J* , **26**, 292 (1932)
17. Beeston, A W., Channon, H. J., and Wilkinson, H., *Biochem. J.*, **29**, 2659 (1935).
18. Best, C. H., Channon, H. J., and Ridout, J. H., *J. Physiol.*, **81**, 409 (1934).
19. Best, C. H , Huntsman, M. E., McHenry, E. W., and Ridout, J. H., *J. Physiol.*, **84**, 38 P (1935).
20 McHenry, E. W., *J. Physiol.*, **85**, 343 (1935).
21. McAmis, A. J , Anderson, W. E., and Mendel, L. B., *J. Biol. Chem.*, **82**, 247 (1929).
22 Burr, G. O., and Burr, M. M., *J. Biol. Chem.*, **82**, 345 (1929).
23. Mitchell, H. K., and Isbell, E. R , *Univ. Texas Pub.*, *No. 4237*, 125 (1942).

STUDIES OF THE UROPORPHYRINS

I. THE PURIFICATION OF UROPORPHYRIN I AND THE NATURE OF WALDENSTRÖM'S UROPORPHYRIN, AS ISOLATED FROM PORPHYRIA MATERIAL[*]

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PLATES 1 AND 2

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The term uroporphyrin was first used by Fischer (1) to designate the ether-insoluble porphyrin occurring in the urine of Petry, the extensively studied case of congenital porphyria (2–6). This substance was not observed in the feces and, until recently (7), was thought to be excreted only in the urine. Coproporphyrin, as isolated from both urine and feces of the case Petry (2), was ether-soluble and contained but four carboxyl groups, while uroporphyrin was found to contain eight (1, 2, 5, 6). Spectroscopic differences were also apparent (3). Partial decarboxylation of uroporphyrin resulted in formation of coproporphyrin (5, 8), although it is noteworthy that the yield was small. Further study of the coproporphyrin thus obtained revealed that its configuration corresponds with etioporphyrin I rather than III, the latter being the isomer type of the protoporphyrin in the hemoglobin molecule (5, 9). This observation led directly to Fischer's postulation of the "dualism of the porphyrins" in nature (5), a concept which has been amply supported by subsequent studies of various investigators (10–18). The four isomeric coproporphyrins were synthesized (9, 19–21). Of these, only Types I and III have been found in nature. The structure of uroporphyrin has not yet been established, the existing evidence (21–23) indicating, however, that it is a tetrapropionic, tetraacetic porphin, as shown in the accompanying formula.

Until 1934 the occurrence of an isomeric uroporphyrin (Type III) had not been reported. In that year Waldenström (24) reported the isolation of a porphyrin possessing uroporphyrin characteristics which upon decarboxylation yielded coproporphyrin III. This new compound was observed to be extracted from the urine by ethyl acetate at a pH of 3.0 to 4.0 (gray reaction to Congo red paper). Its methyl ester was found to melt at 258°. Waldenström (24–26) isolated this porphyrin from the urine of a large number of patients with so called acute porphyria (abdominal or nervous type, in contradistinction to the congenital or light-sensitive form). As a result of Waldenström's studies the natural occurrence of uroporphyrins I and III became generally accepted. It may be noted, however, that the melting point was variously observed between 230–260° (27). Fischer and Hofmann (28) reexamined crystalline uro ester (over 20 years old) from Petry urine by means of a chromatographic method, and reported the presence of a relatively large amount of uroporphyrin I (ester m.p. 302°; corrected, 311°) and a small amount of uroporphyrin III (ester m.p. 261°, not corrected). It is noteworthy that prior to Fischer and Hofmann's report the melting point of uroporphyrin (Type I) had been variously reported between 270–285° (see (29) Table I).

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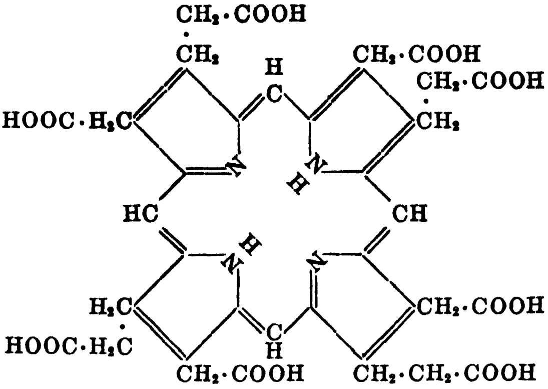

$C_{40}H_{38}O_{16}N_4$

Uroporphyrin I according to Fischer (21)

During the past 6 years two of us (C. J. W. and S. S.) have had opportunity to investigate, in more or less detail, material from fifteen cases of porphyria. This number includes four patients with latent porphyria and one[1] with congenital porphyria (light-sensitive type). The remainder belong to the category which is perhaps best designated as the intermittent acute type, in which abdominal or nervous manifestations are prominent. Some reference to these patients and to isolation of uroporphyrin from their feces has already been made (7, 11, 30). The present communication will deal only with the isolation and purification of uroporphyrin I and of Waldenström's uroporphyrin, as these substances have occurred in certain cases of the series.

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[1] Urine samples from this patient were made available to us through the courtesy of Dr. Peachey and Dr. W. H. Strain of Rochester, New York.

Materials and Methods

The present material included urine from Cases 4, 5, 9, 14, and 15 of the above series, and feces and bile (duodenal drainage) from Case 15. Crystalline porphyrin esters previously isolated from Cases 1, 4, 5, but not previously subjected to chromatographic analysis, were further studied. Thus, uroporphyrin esters isolated from the urine and liver of Case 1 in 1938, from the feces of Cases 4 and 5, and from the urine of Case 5 in 1941, all crystalline, had been kept in the dry state during the intervening period and were available for study. Crystalline uro ester isolated from urine by Grinstein in 1940 (31), in a case of porphyria studied in Cordoba, Argentina, was also available. A small amount of uroporphyrin ester isolated in 1940 by Turner (18) was kindly sent to us by him. As will be noted subsequently, this sample was of particular interest, since it melted at 296° (corrected), a melting point also observed for the same sample in Fischer's laboratory.[2]

All of the chromatographic techniques thus far employed for the isolation of porphyrins from the urine or feces have deviated from the original Tswett method (32) in that so called "flowing" chromatography was used rather than actual division of the column into the component zones of the chromatogram. The "flowing" technique signifies elution of each band separately by means of the same or a series of different solvents. This technique was employed in our earlier studies, with Al_2O_3 or talc as recommended by Waldenström, and by Fischer and Hofmann. Subsequently it was found that precipitated $CaCO_3$, as used originally by Tswett, has several advantages. We first noted its usefulness in purifying protoporphyrin (33). When Zechmeister-Cholnoky tubes (32) were employed, $CaCO_3$ was used for the chromatographic separation of porphyrins from urine, feces, and other sources.[3] Up to the present we have used either Cenco or Merck precipitated $CaCO_3$. While there is no reason to doubt that any pure $CaCO_3$ would be equally satisfactory, it would be necessary to determine the chromatographic behavior for the new brand before conclusions could be drawn. In the present study $CaCO_3$, Cenco, U. S. P. precipitate powder was used throughout. The use of $CaCO_3$, in addition to being most economical, has permitted separation of mixtures hitherto regarded as entities, as will be noted in the following.

The isolation procedure has been simplified considerably. That for the feces has already been described (7) up to the point of the present chromatographic method. The urine is acidified with glacial acetic acid to a pH range of 3.0 to 4.0, after which considerable talc is added. The mixture is shaken thoroughly and the talc, with the adsorbed porphyrin, is collected by filtering on a large Büchner funnel. If insufficient talc is used for the amount of porphyrin present in the sample, then absorption bands of the porphyrin-metal complex (578, 539 mμ) will be observed in the filtrate, in which event it is shaken with more talc and again filtered. The talc is tamped and dried by suction on the funnel, and then removed and powdered, after which it is placed in an Erlenmeyer flask and covered with an excess of methyl alcohol previously saturated in the cold with HCl gas. After standing overnight, the methyl alcohol-HCl is filtered from the talc on a sintered glass filter. The talc is repeatedly washed on the funnel with methyl alcohol-HCl, and finally with $CHCl_3$ until the filtrate is colorless. The comparable extract of the otherwise untreated feces, as previously described (7), is filtered on a Büchner funnel. The filtrate as obtained from either urine or feces is mixed with an equal volume of $CHCl_3$ in a separatory funnel and is then washed repeatedly with distilled water, each time with several volumes. Next it is washed three times with 10 per cent ammonia solution and three times with 7 per cent NaCl solution. It is then filtered through $CHCl_3$-moistened paper, and concentrated to dryness on the water bath. The residue is dissolved in benzene, or, if the amount of porphyrin is small, in benzene with a small amount of petroleum ether. In some instances it is necessary to use a drop of $CHCl_3$ first in order to dissolve the residue in benzene. The petroleum ether increases adsorption on the $CaCO_3$, but has the disadvantage of precipitating the porphyrin if much is present, or if the petroleum ether is used in too high a proportion with the benzene. The porphyrin residues from the feces are more soluble in petroleum ether, presumably owing to the presence of impurities. For these residues, therefore, a benzene-petroleum ether (1:2) mixture was used. In general a mixture of benzene-petroleum ether (3:1) was used for the residues from the urine. The boiling point of the petroleum ether used was 30–60°. Development of the chromatogram from the urine is best effected with benzene alone or with benzene-$CHCl_3$ (10:1). When the urine was used directly, as above, benzene alone was employed in most instances. When porphyrin extracted from the urine with ethyl acetate, according to Waldenström (24–27), was used as starting material, benzene-$CHCl_3$ (10:1 to 10:3) was used. Development of the primary chromatogram of the fecal porphyrins was carried out with benzene-petroleum ether (1:2); the secondary chromatograms, with benzene alone or benzene-$CHCl_3$ (10:1). The details of preparing the column and the chromatogram are exactly as described for the original Tswett method with Zechmeister-Cholnoky tubes (32). The $CaCO_3$ powder is packed by means of suction and tamping with a long wooden pestle. The latter is also used to push the column out of the tube after development of the chromatogram. The individual bands or zones which are cut out of the column are then eluted with $CHCl_3$. The $CHCl_3$ solution is separated from the $CaCO_3$ on a sintered glass filter. Each band is then rechromatographed separately to establish purity. Particular attention has been given to a red-brown zone which has often been noted at the top of the column in the primary chromatograms, and which is not eluted by $CHCl_3$, but may be removed by $CHCl_3$ containing glacial acetic acid (10:2). This porphyrin is thought to have considerable significance and will be considered in more detail later.

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[2] Personal communication from Dr. Turner.

[3] We are grateful to Professor L. Zechmeister, California Institute of Technology, for advice and suggestions with respect to the method used.

The esters crystallize well from $CHCl_3$-methyl alcohol after one chromatography. As already noted, however, rechromatography was usual, especially when two zones were bordering upon one another. In such instances they were cut out as one and rechromatographed.

In four instances (Cases 4, 5, 9, and 15) urine was extracted with ethyl acetate and the porphyrin corresponding to Waldenström's uroporphyrin III was then crystallized. The crystalline uro ester available from the Cordoba case had also been isolated by Waldenström's method (24–27). The methyl ester of this porphyrin, melting at 258–260° (uncorrected), was then dissolved in benzene and subjected to the above ($CaCO_3$) method of chromatographic analysis. The Waldenström porphyrin was adsorbed on the column with benzene-petroleum ether (3:1) and the chromatogram composed, surprisingly, of two rather than one zone was developed by means of benzene-chloroform (10:3). In order to obtain the best separation of these two zones, it was found important not to adsorb a great deal of ester at one time, and to permit adsorption for only about one-sixth of the column, after which development was begun. The chromatogram and the resulting porphyrin entities will be described later.

The method used in 1938 for the isolation of uroporphyrin from the liver of Case 1 may be given briefly here. 538 gm. of fresh liver obtained at necropsy were finely ground with a meat grinder, after which the material was extracted in a mortar with glacial acetic acid and ether to remove hemoglobin and any ether-soluble porphyrins present. This extract contained a moderate amount of porphyrin-metal complex (absorption maxima, I 580, II 542 mμ). The residue was then ground with 10 per cent ammonia. The filtrate of the latter contained much uroporphyrin-metal complex (absorption maxima, I 579, II 542 mμ). This solution was acidified with glacial acetic acid and the porphyrin was collected on talc. After removal from the talc by dilute ammonia the solution was divided in two parts. One-half was treated with HCl to split out the metal, after which sodium acetate was added until a negative reaction with Congo red paper was obtained and the free porphyrin was then collected on talc and concentrated by elution with a small amount of dilute ammonia. On acidification with acetic acid, the uroporphyrin largely precipitated. After the material was dried, the precipitate was esterified with methyl alcohol-HCl and the methyl ester was crystallized from chloroform-methyl alcohol in the usual way. This material was subjected to chromatographic analysis according to the method described by Fischer and Hofmann (28), Brockmann's Al_2O_3 being used as adsorbent, with $CHCl_3$-methyl alcohol and $CHCl_3$ to develop and elute, respectively. The other half of the ammonia solution mentioned above was acidified with acetic acid and the porphyrin-metal complex was again adsorbed on talc. It was then eluted with a small amount of 5 per cent NaOH. An unsuccessful attempt was made to esterify the metal complex by addition of $(CH_3)_2SO_4$. The solution was again acidified and the precipitate was collected and dried, after which the porphyrin was esterified with methyl alcohol-HCl. The ester was extracted with $CHCl_3$, which was then crystallized by the method given above. Some of this material had been kept in the dry state and was available for reexamination during the present study. At this time it was subjected to chromatographic analysis on $CaCO_3$ as already described.

A small amount of bile, obtained from Case 15 by duodenal drainage, was examined with respect to ether-insoluble porphyrins. After acidification with glacial acetic acid and repeated extraction with ether, the aqueous fraction was mixed with talc, and from this point on was treated in the same way as has been described for the urine.

Melting point determinations were carried out routinely with a Fisher-Johns micro melting point apparatus. Two transformers were used in order to permit determinations up to 350°. A calibration curve for the thermometer was prepared with a series of organic compounds of known melting point. This also permitted determination of the corrected melting point. All melting points given in the following are corrected, except where otherwise noted. In addition, macrodeterminations were made in certain instances, olive oil being used as the heating medium.

Spectroscopic measurements in the visible range were made with a Zeiss grating spectrometer, calibrated with the mercury arc. Ultraviolet spectrograms were taken with a Hilger medium quartz spectrograph. Absorption curves were determined with a concentration of 200 γ per 100 cc. We are indebted to Mr. J. F. Marvin, of the Division of Biophysics, for preparing these spectrograms.

Decarboxylations were carried out in 1 per cent HCl in sealed tubes for 3 hours at 180–190°, in accordance with Fischer's method (21). The HCl solution was neutralized with sodium acetate and the porphyrins were extracted with ether. They were then taken into a small amount of 10 per cent HCl from which they were precipitated by addition of sodium acetate until a negative reaction was obtained with Congo red paper. The precipitate was collected, dried, and esterified in methyl alcohol-HCl. After the usual purification of the $CHCl_3$ solution, as described in the foregoing, the further treatment was as follows: The material from the 284° porphyrin (see below) of Case 15 was crystallized directly. After three recrystallizations the melting point was constant between 210–216°. These crystals were then chromatographed as already described. The $CHCl_3$ solutions of the esters obtained after decarboxylation of the 284° porphyrins of Cases 4 and 5, and from the Cordoba case, were subjected directly to chromatography without being first crystallized. The 208° porphyrins (see below) from Case 15 and from the Cordoba case were also subjected to decarboxylation. An attempt was made to crystallize the products of the reaction but the yield was so small that the crystals were returned to the mother liquor, and after concentration to dryness the entire residue was redissolved in benzene and chromatographed. The porphyrins from each decarboxylation were adsorbed on $CaCO_3$ from benzene-petroleum ether (1:1), and the chromatogram was developed by benzene-$CHCl_3$ (20:1).

Microanalyses for carbon and hydrogen were carried out in the laboratories of the Division of Organic Chemistry. The nitrogen[4] and the micromethoxyl determinations were made by Dr. Hayman through the courtesy of the Merck Laboratories in Rahway, New Jersey.

Various metal complexes of the porphyrins isolated were prepared by means of a slight modification of Fischer's method (21). The chloroform solution of the porphyrin ester is mixed with a small amount of the metal acetate dissolved in glacial acetic acid. The mixture is heated briefly in the boiling water bath. In the case of the copper complex crystallization occurs upon addition of methyl alcohol and further brief heating. The zinc, cobalt, and silver complexes crystallize poorly in the presence of much acetic acid. The chloroform solutions were therefore washed repeatedly with water, filtered through chloroform-moistened paper, and concentrated to a small volume. Crystallization was then brought about by addition of methyl alcohol and further heating. In some instances a small amount of free ester was separated from the metal complex by chromatographic analysis on $CaCO_3$. Adsorption was effected with benzene and development with benzene-chloroform (10:2). The metal complex is characterized by a rose band high on the column, the free ester by a red-violet band considerably lower. Of importance to later considerations, it may be noted that we have not been able to separate metal complex from free porphyrin ester on a column of talc, developed with $CHCl_3$, according to the procedure of Fischer and Hofmann (28).

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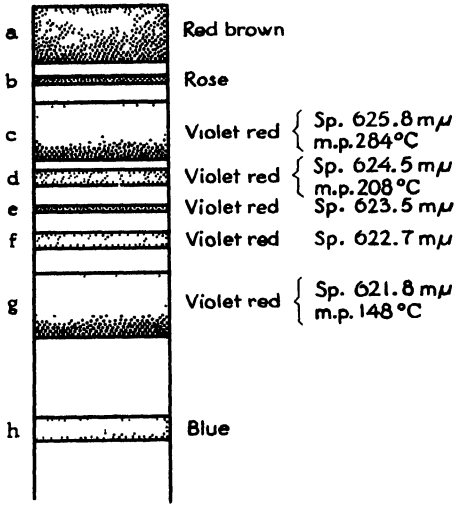
[4] The samples were burned in the presence of copper acetate, according to the method of Hayman and Adler (34).

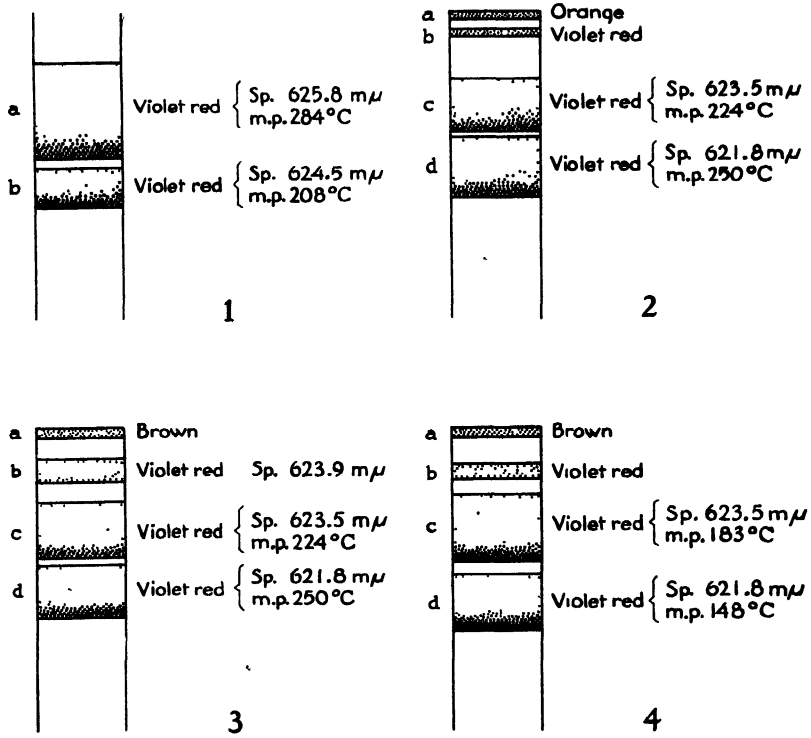
Results

In the primary chromatogram (Text-fig. 1) containing all of the substances collected by talc from the urine of Case 9 as described above, it is seen that, just as in the case of carotenoids and other compounds (32), a close relationship exists between the color and absorption spectrum of the porphyrin on the one hand, and the adsorbability on the other. The greater the adsorbability (higher location of the zone on the column) the farther is the shift of the absorption bands to the left. Slight differences in absorption are best noted in the band found in the red region, that with absorption in the region of 625 mμ. As seen in Text-fig. 1, a complex mixture of pigments occurs in porphyria urine. The substances composing zones *a*, *b*, and *h* were not porphyrins and were not further identified. Zones *c* and *d* are of most importance and will be discussed below. Zones *e* and *f* are unidentified porphyrins. These have been encountered repeatedly in chromatograms from the urine of other patients, including Cases 4, 5, and 15. Zone *g* is the coproporphyrin zone which in Case 9 (Text-fig. 1) was shown to be coproporphyrin III. In other cases the proportion of the porphyrins varied from that shown in Text-fig. 1, as will be discussed in the following.



TEXT-FIG. 1. Diagram of the chromatographic separation of porphyrin esters and other pigments from the urine of Case 9. Sp. (spectrum) indicates the midpoint of the absorption band in the red, of the dissolved porphyrin ester. Zone *c* is the 284° porphyrin (uroporphyrin I), zone *d* the 208° porphyrin, the two together having originally comprised the Waldenstrom, or 260° porphyrin. Zone *g* is that of coproporphyrin III methyl ester, which in this case was present in considerable amount (see the text).

The porphyrin ester isolated according to Waldenström's method, from Case 15 and the Cordoba case, was found to have a melting point of 258–262°, constant after repeated recrystallization. The two porphyrins obtained by chromatographic analysis of these crystals exhibit closely set



TEXT-FIG. 2. Diagrams of chromatograms of porphyrin esters and other compounds from various urine samples. Chromatogram 1, separation of the Waldenström porphyrin into the 284° and 208° porphyrins, as observed in Case 15 and the Cordoba case; Chromatogram 2, separation of the compounds comprising the crystalline material melting at 210–216° and obtained by decarboxylation of the 284° porphyrin from Case 15; Chromatogram 3, separation of the decarboxylation products from the 284° porphyrin, as observed in Cases 4 , 5, and 15, and the Cordoba case; Chromatogram 4, separation of the decarboxylation products from the 208° porphyrin, as observed in Case 15 and the Cordoba case.

zones on the chromatogram, the upper of which is usually 3 times larger than the lower (Text-fig. 2, Chromatograph 1). These are identical with zones *c* and *d* as shown in Text-fig. 1. When dissolved separately in chloroform it is seen that the absorption band of the upper zone nearest the red region is maximum at 625.8 mμ (in $CHCl_3$), that of the lower at

624.5 mμ (in $CHCl_3$). The former crystallizes promptly from $CHCl_3$-MeOH in long needles melting at 284°. The porphyrin from the lower zone crystallizes slowly, on cooling, in rosettes of very thin and often bending needles melting at 208°. The $CHCl_3$ must be boiled off as completely as possible. Once pure, both of these esters are insoluble in methyl alcohol. Rechromatographed separately, each exhibits but a single zone. The crystals of the initial uroporphyrin ester obtained according to Waldenström, but prior to $CaCO_3$ chromatography, may be compared with those of its two components in Figs. 1 to 3. For purposes of discussion these two porphyrins will be referred to in the following according to their melting points; *i.e.*, 208° and 284°. Both of these melting points have been confirmed repeatedly by means of macro as well as micro methods. Sufficient amounts of each of the two were isolated from the urine of Case 15, and from the crystalline material of the Cordoba case, to permit comparison of relative amount, as shown in Table I.

TABLE I

Relative Amounts of Porphyrin Methyl Esters with Melting Points of 208° and 284°, As Isolated from Urine of Case 15 and Cordoba Case

Case No.	Amount			
	208°	284°	208°	284°
	mg	*mg*	*per cent*	*per cent*
15	11.0	29.0	27.5	72.5
Cordoba (C. P. T.)	12.0	31.0	28	72

The amounts of the 208° and 284° porphyrins obtained from Cases 4 and 9 and of the 284° porphyrin from Case 5 were considerably smaller and were not weighed. Judging by the size and color intensity of the zones on the chromatogram, the relative amounts obtained from Case 4 were in the same proportion as shown in Table I. In Case 9, however, the 284° porphyrin was present in considerably larger proportion, estimated roughly at 5:1 by the chromatogram (see Text-fig. 1). It is of interest that this patient excreted relatively large amounts of coproporphyrin III, ranging over a period of time at about 4:1 of the ether-insoluble porphyrins. The coproporphyrin III from this patient was isolated readily after chromatographic analysis, as shown in Text-fig. 1 (zone *g*). The crystals of the methyl ester are shown in Fig. 8. The melting point was 148°. In the case of congenital porphyria (Case 8 in the present group) the urine contained only the 284° porphyrin.

The properties of the two porphyrins, 208° and 284°, were the same for the various patients from which they were isolated. The 284° porphyrin methyl ester is characterized by the following absorption maxima in $CHCl_3$:

I 625.8, II 599.0, III 571.0, IV 535.7, V 503.4 mμ; order of intensity V, IV, III, I, II. The absorption maxima of the free porphyrins in 25 per cent HCl are I 597.0, II 553.8, and III 410.3 mμ (III, II, I). The corresponding maxima for the 208° porphyrin are (for the ester in $CHCl_3$) I 625.0, II 598.0, III 569.9, IV 534.6, V 500.8 mμ; (for the free porphyrin in 25 per cent HCl) I 595.8, II 552.6, III 408.7 mμ (III, II, I). The extinction coefficient for the 208° porphyrin was approximately 3×10^{-6}.

The 208° porphyrin in ethyl acetate exhibits the following absorption maxima, I 624.5, II 597.0, III 568.8, IV 531.7, V 498.5 mμ; order of intensity V, IV, I, III, II. It may be emphasized that although the Waldenström porphyrin melting at 258° is extracted from the urine by ethyl acetate at pH 3.0 to 4.0, its two components behave quite differently toward this solvent, once pure. The 284° porphyrin is insoluble, while the 208° porphyrin is easily extracted from aqueous solution even when negative to Congo red paper (although acid in reaction).

The results of the elementary analyses were as follows:

284° Porphyrin—

Uroporphyrin octamethyl ester, $C_{48}H_{54}O_{16}N_4$.	Calculated,	C	61.12;	found,	60.90
	"	H	5.77;	"	5.56
	"	N	5.94;	"	5.94
	"	OCH_3	26.3;	"	26.72

208° Porphyrin—

	Uroporphyrin octamethyl ester	Heptamethyl ester	Found
C	61.12	62.41	61.70, 61.96
H	5.77	5.93	5.77, 6.02
N	5.94	6.34	6.52
OCH_3	26.3	23.0	22.82

The properties of the metal complexes of the 284° and 208° porphyrin esters are given in Table II, and the crystals are shown in Figs. 10 to 16.

As noted previously, particular attention was given to the red-brown zone appearing at the top of the primary chromatogram. This was noted in Case 5 (urine) and in the studies of the crystals previously isolated from Cases 4 and 5. In all instances most of this zone could be eluted only in $CHCl_3$ containing glacial acetic acid. After reesterification, however, the behavior on the column was that of the 284° porphyrin, and, when crystallized, all of the properties of this porphyrin were shown. In Case 5 a very small amount of the red-brown zone was eluted with $CHCl_3$ and when crystallized melted at 284°. The major portion was eluted with $CHCl_3$ containing glacial acetic acid and was then crystallized from $CHCl_3$-methyl alcohol, after removal of acetic acid by washing with ammonia. The crystals, composed of small needles, melted at 261° (shrinking above 245°).

After reesterification in methyl alcohol-HCl, the porphyrin ester behaved exactly like the 284° porphyrin except that in this instance the melting point could not be elevated above 272°. The absorption spectrum was identical.

Results of Decarboxylation of 208° and 284° Porphyrins

Decarboxylations were carried out on the 208° and 284° porphyrins from Case 15 and the material from the Cordoba case, and on the 284° porphyrin from Cases 4 and 5. As already noted under the methods, the porphyrin esters obtained following decarboxylation of the 284° porphyrin from Case 15 exhibited a constant melting point of 210–216° after three crystallizations. These crystals appeared to be uniform in type, consisting of yellowish red prisms, often occurring in rosettes (Fig. 4). Upon chromatography it was found that four zones resulted (Text-fig. 2, Chromatogram 2). Zones *a* and *b* were small in amount and were not identified further except that zone *b* was a porphyrin, while zone *a* was not. Zones *c* and *d* are identical with *c* and *d* in Chromatogram 3 (Text-fig. 2), derived by direct chromatography, as described under the methods. As noted below, zone *d* was identified as coproporphyrin I. This was obviously purer than the coproporphyrin I obtained from the corresponding zones of the chromatograms from Cases 4 and 5, and the Cordoba case, in which the porphyrins were chromatographed directly without previous crystallization, as described for Case 15. In the latter instance the coproporphyrin I methyl ester (Fig. 5) melted at 248–250° after two recrystallizations, while that from the other three patients melted over a longer range (235–244°) even after several recrystallizations. It appeared that the crystallizations prior to chromatography in Case 15 removed certain impurities retained on the chromatograms in the other instances.

TABLE II

Melting Points and Absorption Maxima of Metal Complexes of 208° and 284° Porphyrin Esters

Porphyrin		Absorption maxima	M p
		mμ	*°C*
284°	Cu	564.8, 527.9	319 (Uncorrected, decomposition)
	Zn	574.7, 539.1	330 “ “
	Co	554.3, 519.2	316 “ “
	Ag	562.6, 528.3	306 “ “
	Cd	Not measured	Above 330
208°	Cu	564.6, 527.7	277–283
	Zn	574.6, 538.8	267–274
	Co	554.6, 518.4	268–275
	Ag	562.7, 528.2	249–257

The porphyrin ester of zone *c* (Chromatograms 2 and 3 in Text-fig. 2) crystallized easily from methyl alcohol in rather characteristic curving needles, often in rosettes (Fig. 6). When recrystallized the following properties were exhibited.

Melting Point—221–224°.

Absorption Maxima in $CHCl_3$—I 623.5, II 568.1, III 535.5, IV 501.2 mμ (IV, III, II, I).

Absorption Maxima in 25 Per Cent HCl—I 594.6, II 550.4, III 405.9 mμ (III, II, I).

Absorption Maxima of Free Porphyrin in Ethyl Ether Containing Acetic Acid—I 624.3, II 568.3, III 530.0, IV 497.5 mμ (IV, III, I, II).

The HCl number of the porphyrin was found to approximate 0.1 per cent (most of the porphyrin dissolved in ether being extracted from it by an equal volume of 0.1 per cent HCl).

The yield of crystalline ester of the 224° porphyrin from Case 15 was 0.5 mg. from 8 mg. of the 284° porphyrin. From 5 mg. of the 284° porphyrin isolated from the Cordoba case, 1.2 mg. were obtained. Elementary analysis was carried out on a mixture of the material obtained from the four patients noted above. The results are as follows:

Pentamethyl ester, $C_{42}H_{48}O_{10}N_4$. Calculated, C 65.63; found 66.08
" H 6.25; " 6.29

Further decarboxylation resulted in partial formation of coproporphyrin I, identified by the methyl ester melting point (245°) and characteristic absorption spectrum.

The properties of the metal complexes of the 224° porphyrin ester were found to be as follows:

	M.p °C	Absorption maxima mμ
Cu	266–269	I 563.4, II 526.2 (I, II)
Zn	282–286	" 572.6, " 536.5 " "
Ag	249–254	" 560.9, " 526.1 " "

Zone *d* in Chromatogram 3, Text-fig. 2, crystallized in the typical needles of coproporphyrin I methyl ester and exhibited all of the properties of this porphyrin. The yield from Case 15 was 2.1 mg. from 8 mg. of the 284° porphyrin. From the Cordoba case 0.9 mg. was obtained from 5 mg. The melting point was 248–250°. The absorption maxima were (in 25 per cent HCl) I 592.7, II 548.9 mμ; in order of intensity, II, I. The absorption of the free porphyrin in ether and acetic acid was I 623.4, II 567.8, III 528.1, IV 495.6 mμ; IV, III, I, II. The HCl number was less than 0.1 per cent. The Cu complex of the methyl ester melted at 280–285°. The absorption maxima were I 562.0, II 524.6 mμ; I, II.

Decarboxylations of the 208° porphyrins from Case 15 and the Cordoba case yielded identical results in both instances. The chromatogram of the products of decarboxylation is shown in Text-fig. 2, Chromatograph 4. Zones *a* and *b* were not further identified except that zone *b* was a porphyrin, and zone *a* was not. The porphyrin ester of zone *c* crystallized in poorly formed, clumping needles (Fig. 9). The mother liquor remained relatively colored, in contradistinction to the zone *d* porphyrin (coproporphyrin III), the mother liquor of which was almost colorless. The other properties of the zone *c* porphyrin are as follows: m.p. 180–183°; absorption maxima of the ester in $CHCl_3$, I 623.1, II 567.5, III 535.4, IV 500.8 mμ; order of intensity, IV, III, II, I. The free porphyrin in 25 per cent HCl exhibits the following absorption maxima, I 594.6, II 549.8, III 406.2 mμ; III, II, I; in ether-acetic acid, I 623.6, II 568.8, III 529.8, IV 495.5 mμ; IV, III, I, II. The HCl number of this porphyrin was less than 0.1 per cent. The metal complexes of the ester had the following properties.

	M p. °*C*.	Absorption maxima mμ
Cu	214–223	I 563 8, II 527.2 (I, II)
Ag	204–211	" 561.0, " 525.9 " "
Zn	248–253	" 555 6, " 527.7 " "

The zone *d* porphyrin crystallized readily in the form of rosettes of prisms (Fig. 7). The melting point was 148°. After cooling, the crystals which formed again melted at 170° (dimorphic melting point). The crystals were identical in appearance with those of the coproporphyrin III methyl ester isolated from the urine of Case 9, the chromatogram of which is seen in Text-fig. 1 (zone *g*), and the crystals in Fig. 8. These crystals likewise melted at 148°. A mixture of the crystals of these two coproporphyrins gave but a single zone on a $CaCO_3$ column. The crystals obtained from this zone melted at 148°, this constituting a mixed melting point without depression. The absorption spectra were identical with that of coproporphyrin.

The properties of the copper complex of the coproporphyrin III (zone *d*) ester were as follows: m.p. 206–210°; absolute maxima in $CHCl_3$; I 562.2, II 524.8 mμ; I, II.

The uroporphyrin I methyl ester sent to us by Dr. W. J. Turner melted at 297°. The absorption maxima in $CHCl_3$ were I 626.0, II 565.9, III 528.3, IV 500.0 mμ; order of intensity, II, III, IV, I. It was obvious that the majority was in the form of a metal complex. The chromatogram on $CaCO_3$ exhibited four zones. The upper two were faint, insufficient to identify. The third, or main zone, was rose in color, and the absorption maxima in $CHCl_3$ were I 566, II 528.5 mμ; I, II. The melting point of this zone was 316° (with decomposition). The fourth zone was red-violet and exhibited absorption identical with the 284° porphyrin (uroporphyrin I) methyl ester. The amount was too small to crystallize. Another portion of the original material was chromatographed on talc, according to Fischer and Hofmann's method (28). Two zones developed corresponding closely to those noted by Fischer and Hofmann in their talc chromatogram of Petry uroporphyrin ester, the results of which induced them to give the melting point of uroporphyrin I methyl ester as 311° (corrected). It is noteworthy that they did not state the absorption spectrum of the rose zone from which the compound with this melting point was obtained. In the present instance the absorption of the rose zone was I 626.0 (very faint), II 566.0, III 528.0, IV 500.0 mμ; II, III, IV, I. Bands II and III were intense, clearly those of a metal complex. The methyl ester of this metal complex, as obtained from the $CaCO_3$ chromatogram, melted at 316° (not corrected), and was dissolved in concentrated H_2SO_4 to split out the metal (probably Cu). After reesterification the absorption spectrum in $CHCl_3$ and the crystals from methyl alcohol were identical with those of the 284° porphyrin. The melting point was slightly higher at 287°.

The uro ester isolated from the urine of Case 1 in 1938 melted then at 289° (uncorrected; prior to chromatographic analysis). Fischer and Hofmann's method was used at that time to separate the porphyrin into two zones on talc. The first remained at the top of the column as a rose-red zone, insoluble in $CHCl_3$ but soluble in glacial acetic acid. The second came well down on the column and was eluted with $CHCl_3$. The crystals of the first zone melted at 296–298° (uncorrected). The crystals of the lower, $CHCl_3$-soluble, zone melted at 286–288° (uncorrected). During the present investigation a small amount of the 296°, or upper zone, porphyrin was reexamined. The crystals were quite insoluble in $CHCl_3$. They did not melt up to 345°, although some darkening was noted above 275°. Because of general insolubility, chromatographic analysis was impossible. This behavior was not explained but was thought to be due to some change which had occurred since 1938. The fact that the melting point at that time was 296° may also have been due to the presence of metal complex. The absorption maxima of this fraction in 1938 were I 626.5, II 569.0, III 534.0, IV 501.0 mμ (in $CHCl_3$ in which, at that time, the material was sparingly soluble). This undoubtedly contained metal complex, while the lower, $CHCl_3$-soluble, zone was composed at least largely of free ester with absorption maxima of I 626.3, II 575.8, III 536.0, IV 505.0 mμ.

Feces, Liver, and Bile Porphyrins

The 208° porphyrin was isolated from the feces of Case 15. So far as could be determined, these feces did not contain any of the 284° porphyrin. Crystalline porphyrin ester previously isolated from the feces of Case 4 (7), melting in 1941 at 208° (uncorrected), was now found to be an entity on the $CaCO_3$ column. After crystallization a melting point of 208° (corrected) was observed. The absorption spectrum was identical with that of the 208° porphyrin, as already given. It is of particular interest that the 208° porphyrin was not observed either in the feces or urine of Case 5. This was true of several samples of both from different periods.

A crystalline uroporphyrin ester previously isolated from the feces of Case 5 (7), melting in 1941 at 280° (uncorrected), was also found to be an entity by $CaCO_3$ chromatography. After crystallization at this time, however, the corrected melting point was 294°. The type of crystal and the spectroscopic properties were identical with those of the 284° porphyrin. The elevation of the melting point to 294° in this instance is not explained. It is believed that older samples may suffer slight changes affecting the melting point in one way or another. Thus, crystals isolated from the urine of Case 4 in 1940, kept in a soft glass tube in the interim, and now chromatographed on $CaCO_3$, yielded a zone behaving like the 284° porphyrin but melting at 270–272°. Urine from this individual, which had been stored in the ice box, yielded a porphyrin identical in other respects, whose ester melted at 284°. In Case 5, however, the melting point could not be elevated above 272°, either from urine or crystalline material stored since 1941, and this was true in spite of recrystallization, rechromatography, saponification and extraction with ethyl acetate, and reesterification.

The bile-containing duodenal contents of Case 15 contained an ether-insoluble porphyrin which exhibited the absorption spectrum and chromatographic behavior of the 208° porphyrin. The amount was insufficient to crystallize.

It may be noted that dithizone analysis[5] of the uroporphyrin-metal complex from the urine and liver of Case 1 revealed that the metal was zinc.

The uroporphyrin ester obtained from the liver of Case 1 in 1938, by means of the chromatographic method of Fischer and Hofmann, exhibited two zones on the talc column. About 4 mg. of crystals were obtained from the upper of these zones with a melting point of 279–281° (not corrected). 3 mg. were obtained from the lower zone; these melted at 275–277° (not corrected). After decarboxylation of the latter, coproporphyrin I methyl ester was isolated with a melting point of 244–245° (not corrected). The only sample available for reexamination during the present study was that obtained after an unsuccessful attempt to esterify the zinc complex with $(CH_3)_2SO_4$. These crystals melted in 1938 at 271–274° (not corrected). Upon $CaCO_3$ chromatography in 1943 four zones were noted, two of which were very faint and not identified. The main zone behaved like that of the 284° porphyrin and the absorption was identical; the corrected melting point, however, was but 268°. A smaller zone, lower on the column, and exhibiting the absorption of the 208° porphyrin, melted at 207°.

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[5] This was carried out through the courtesy of Dr. E. B. Sandell, Division of Analytical Chemistry, University of Minnesota.

DISCUSSION

The use of Tswett columns of calcium carbonate has permitted what is believed to be a complete purification of uroporphyrin I octamethyl ester, m.p. 284°. The present evidence indicates that variant melting points observed in the past were too high because of partial saponification or presence of metal complex, or too low because of varying amounts of the 208° porphyrin. It is true that these factors are insufficient to explain all of the higher or lower melting points noted in the present study. Old samples, even after reesterification, rechromatography, and recrystallization, may melt above or below 284°. Since the physical characteristics of these samples are otherwise identical, the reason for the variation is not clear.

It has been shown in the present study that the 258–260° porphyrin obtained from porphyria urine by Waldenström's method of ethyl acetate extraction may often[6] be separated on the Tswett column into two components, uroporphyrin I octamethyl ester with a melting point of 284°, and a heptamethyl ester with a melting point of 208°. The latter is ether-insoluble and in other respects similar to uroporphyrin. In this connection it is of interest to note that Waldenström's methoxyl percentage for the 258° porphyrin was somewhat too low (25). The 208° porphyrin undoubtedly corresponds in configuration with etioporphyrin III, so that it would be permissible to designate it as uroporphyrin III except that, according to the present evidence, it is not isomeric with uroporphyrin I. The question may be raised as to why the porphyrin extracted by ethyl acetate by Waldenström's method has exhibited such a constant ester melting point of 258° (24–26, 29). This is believed due to the fact that the methyl esters of the 284° and 208° porphyrins are quite insoluble in methyl alcohol, once pure. The possibility of a loose molecular compound between the two must also be kept in mind, and will be considered further in Paper II, which follows. It is not surprising that a difference in absorption spectrum was not established, since the 258° porphyrin of Waldenström in our experience consists of more than 50 per cent uroporphyrin I whose absorption maxima are but 12 A higher than those of the 208° porphyrin.

Further studies are needed with respect to the proportion of the 284° and 208° porphyrins occurring in the urine, feces, and organs of porphyria cases. In the present study it was of particular interest that the 208° porphyrin was not observed in Case 5, a typical example of intermittent acute porphyria with paralysis and abdominal colic. Nor was it found in the urine of the congenital porphyria case. In Case 9 the 284° porphyrin (uroporphyrin I) predominated in the urine over the 208°, or Type III porphyrin, but at the same time a rather large amount of coproporphyrin III was excreted. The significance of this is not clear, but it would obviously be of much interest to obtain quantitative data on this point in future cases of the disease. It is evident from the present results that uroporphyrin I (284°) is regularly excreted, at least in the urine, and that any classification of porphyria based upon excretion of uroporphyrin I or uroporphyrin III is certain to be misleading.

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[6] Additional cases have been studied more recently in which the initial ester melting point, before chromatographic analysis, was between 250–270°, but in which the 208° porphyrin was not encountered in the chromatogram. The findings in these instances will be considered in Paper II, which follows.

Waldenström (25) and Mertens (29) both obtained coproporphyrin III by decarboxylation of the 258° porphyrin, as extracted from the urine by ethyl acetate. In view of the present finding that uroporphyrin I regularly constitutes the majority of the 258° porphyrin, the question may be asked as to why coproporphyrin I was not obtained, at least in part. The present study reveals that upon decarboxylation of the 258° porphyrin the crystals first obtained and apparently representing an entity melted at 160–162°, which might readily have been accepted as indicating coproporphyrin III, especially since the ether solubility and absorption maxima were now characteristic. Upon chromatography, however, this material was found to be a mixture of three porphyrins not over 10 per cent of which was actually coproporphyrin III, the main portion consisting of the 224° porphyrin, probably a pentamethyl ester, and, as already noted, obtained regularly after decarboxylation of uroporphyrin I. Coproporphyrin I is obtained upon decarboxylation of the 224° porphyrin. Thus it is quite possible that the melting points construed by Waldenström and Mertens as indicating coproporphyrin III may have been melting points of a mixture of decarboxylation products, including coproporphyrin III and the 183° porphyrin derived from the 208° porphyrin, coproporphyrin I and the 224° porphyrin derived from uroporphyrin I. Any combinations of these are possible and may well account in part for the variety of melting points for coproporphyrin III found in the literature, ranging from 135–180°.

SUMMARY

1. The porphyrin described by Waldenström as uroporphyrin III is extracted from urine by ethyl acetate at a pH of 3.2 to 3.4. The melting point of the methyl ester of this porphyrin has been noted by Waldenström and others to be 258–260°. In the present study it has been shown that this porphyrin consists of uroporphyrin I (melting point of the methyl ester, 284°) and a hitherto undescribed porphyrin whose methyl ester melts at 208°. Elementary analysis indicates that the latter is a heptamethyl ester, and consequently not an isomer of uroporphyrin I.

2. The relative constancy of melting point of the Waldenström porphyrin methyl ester (258–260°) after repeated recrystallization from chloroform-

methyl alcohol is probably due at least partly to the insolubility in methyl alcohol of its two components; *i.e.*, the 284° (uroporphyrin I) and the 208° porphyrins. The possibility of a molecular compound is considered.

3. Uroporphyrin I and the 208° porphyrin exhibit but slightly different absorption spectra, the maxima of the former being 12 A toward the red from those of the latter.

4. Decarboxylation of the Waldenström porphyrin often results in a primary mixture of ether-soluble porphyrins having an absorption spectrum of the coproporphyrin type. This mixture melts at 160–162°. It is composed of at least four substances, of which about 10 per cent is coproporphyrin III. A considerable fraction is composed of a porphyrin whose methyl ester melts at 221–224°. This is probably a pentamethyl ester. It is regularly obtained together with coproporphyrin I by means of calcium carbonate chromatography of the decarboxylation products of uroporphyrin I. The 224° porphyrin may be further decarboxylated to coproporphyrin I.

5. The wide range of melting points previously reported for uroporphyrin I methyl ester (265–311°) is explained in part by the presence of partially saponified material, or metal complex, both of which elevate the melting point, or by the presence of the 208° porphyrin which lowers it. Unexplained discrepancies in melting point, in old samples, both above and below 284°, have also been noted.

6. The present findings fail to indicate the occurrence in porphyria material of a uroporphyrin (Type III) isomeric with the Type I uroporphyrin.

BIBLIOGRAPHY

1. Fischer, H., *Z. physiol. Chem.*, **95,** 34 (1915), **98,** 78 (1916–17), **97,** 109 (1916).
2. Fischer, H., *Z. physiol. Chem.*, **96,** 148 (1915–16), **98,** 14 (1916–17).
3. Fischer, H., and Zerweck, W., *Z. physiol. Chem.*, **138,** 176, 242 (1924).
4. Fischer, H., Hilmer, H., Lindner, F., and Putzer, B., *Z. physiol. Chem.*, **150,** 44 (1925)
5. Fischer, H., and Treibs, A., in Oppenheimer, C., Handbuch der Biochemie des Menschen und der Tiere, Jena, 2nd edition, suppl., 72 (1930).
6. Borst, M., and Königsdörffer, H., Untersuchungen uber Porphyrie, Leipzig (1929).
7. Schwartz, S., and Watson, C. J., *Proc. Soc. Exp. Biol. and Med.*, **47,** 390 (1941).
8. Fischer, H., and Hilger, J., *Z. physiol. Chem.*, **149,** 65 (1925)
9. Fischer, H., Platz, K., and Morgenroth, K., *Z. physiol. Chem.*, **182,** 265 (1929).
10. Watson, C. J., *J. Clin. Invest.*, **14,** 106, 110, 116 (1935); **15,** 327 (1936); **16,** 383 (1937), Symposium on the blood and blood-forming organs, Madison, 14 (1939).
11. Watson, C. J., *South. Med. J.*, **36,** 359 (1943).
12. Dobriner, K., *J. Biol. Chem.*, **113,** 1 (1936); *Proc. Soc. Exp. Biol. and Med.*, **35,** 175 (1936).
13. Dobriner, K., and Rhoads, C. P., *J. Clin. Invest.*, **17,** 95, 105 (1938); *Physiol. Rev.*, **20,** 416 (1940).
14. Rimington, C., *Compt.-rend. trav. Lab. Carlsberg*, **22,** 454 (1938); *Proc. Roy. Soc. Med.*, **32,** 1268 (1939).

15. Chandler, F. S., Harrison, S. A , and Rimington, C., *Brit. Med. J.*, **2**, 1173 (1939).
16. Grotepass, W., *Z. physiol. Chem.*, **252**, 155 (1938); **253**, 276 (1938).
17. Turner, W. J., and Obermayer, M. D , *Arch. Dermat. and Syphilol.*, **37**, 549 (1938).
18. Turner, W. J., *Arch. Int. Med* , **61**, 762 (1938).
19. Fischer, H., and Andersag, H., *Ann. Chem.*, **458**, 117 (1927).
20. Fischer, H., and Hierneis, J., *Z. physiol. Chem.*, **196**, 155 (1931).
21. Fischer, H., and Orth, H , Die Chemie des Pyrrols, Pyrrolfarbstoffe, 1. **Hälfte,** Leipzig (1937).
22. Fischer, H., and von Holt, E., *Z. physiol. Chem.*, **227**, 124 (1934).
23. Fischer, H., and von Holt, E., *Z. physiol. Chem.*, **229**, 93 (1934).
24. Waldenström, J , *Acta med. Scand.*, **83**, 281 (1934).
25. Waldenstrom, J., *Deut Arch. klin. Med.*, **178**, 38 (1935).
26. Waldenstrom, J., *Z. physiol. Chem.*, **239**, suppl. 3 (1936); *Acta med. Scand.*, suppl. (1937).
27. Waldenstróm, J , Fink, H., and Hoerburger, W., *Z. physiol. Chem.*, **233**, 1 (1935).
28. Fischer, H., and Hofmann, H., *Z. physiol. Chem* , **246**, 15 (1937).
29. Mertens, E., *Z. physiol. Chem.*, **250**, 57 (1937).
30. Watson, C. J., and Schwartz, S , *Proc. Soc Exp Biol. and Med.*, **47**, 393 (1941).
31. Grinstein, M., *Prensa méd. argentina*, **27**, 2265, 2312 (1940).
32. Zechmeister, L , and Cholnoky, L., Principles and practice of chromatography, translated from 2nd German edition by Bacharach, A. L., and Robinson, F. A., New York (1943).
33. Grinstein, M., and Watson, C. J., *J. Biol. Chem.*, **147**, 667 (1943).
34. Hayman, D. F., and Adler, S., *Ind. and Eng. Chem* , *Anal. Ed.*, **9**, 197 (1937).

EXPLANATION OF PLATES

Plate 1

Fig. 1. Crystals of the Waldenström or 260° porphyrin ester, prior to chromatographic analysis. The magnification = 150 × throughout.

Fig. 2. Crystals of the 284° porphyrin (uroporphyrin I methyl ester).

Fig. 3. Crystals of the 208° porphyrin (heptamethyl ester).

Fig. 4. Crystals of the porphyrin mixture exhibiting a constant melting point of 210–216°, obtained following decarboxylation of the 284° porphyrin.

Fig. 5. Crystals of coproporphyrin I methyl ester obtained by decarboxylation of the 284° porphyrin (Case 15) and subsequent chromatographic separation.

Fig. 6. Crystals of the 224° porphyrin (pentamethyl ester) as obtained by decarboxylation of the 284° porphyrin, with subsequent chromatographic separation.

Fig. 7. Crystals of coproporphyrin III methyl ester (m.p. 148°, 170°) obtained by decarboxylation of the 208° porphyrin and subsequent chromatographic separation.

Fig. 8. Crystals of coproporphyrin III methyl ester (m.p. 148°) as isolated from the urine of Case 9.

Plate 2

Fig. 9. Crystals of the 183° porphyrin methyl ester obtained by decarboxylation of the 208° porphyrin. The magnification = 150 × throughout.

Fig. 10. Crystals of the copper complex of the 284° porphyrin methyl ester (uroporphyrin I).

Fig. 11. Crystals of the copper complex of the 208° porphyrin methyl ester

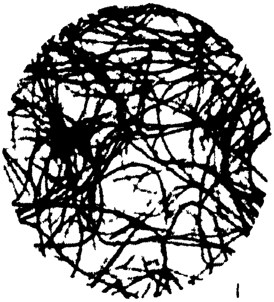
Fig. 12. Crystals of the silver complex of the 284° porphyrin methyl ester (uroporphyrin I).

Fig. 13. Crystals of the silver complex of the 208° porphyrin methyl ester.

Fig. 14. Crystals of the silver complex of the 224° porphyrin methyl ester.

Fig. 15. Crystals of the zinc complex of the 284° porphyrin methyl ester.

Fig. 16. Crystals of the cobalt complex of the 284° porphyrin methyl ester



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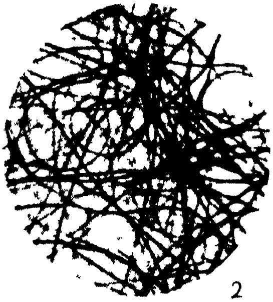

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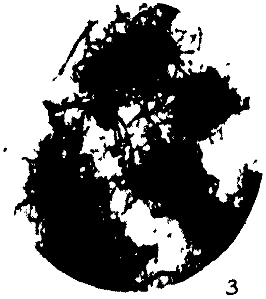

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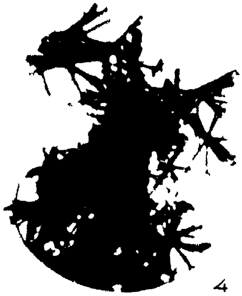

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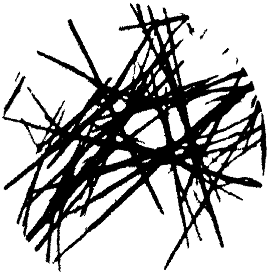

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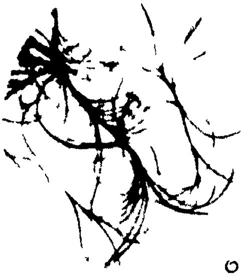

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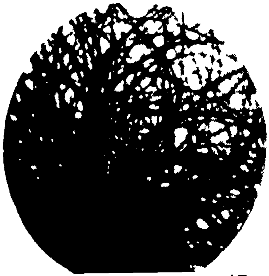
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(Grinstein, Schwartz, and Watson Uroporphyrins. I)

STUDIES OF THE UROPORPHYRINS

II. FURTHER STUDIES OF THE PORPHYRINS OF THE URINE, FECES, BILE, AND LIVER IN CASES OF PORPHYRIA, WITH PARTICULAR REFERENCE TO A WALDENSTRÖM TYPE PORPHYRIN BEHAVING AS AN ENTITY ON THE TSWETT COLUMN*

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The term Waldenström porphyrin is used to refer to a porphyrin extracted from the urine by ethyl acetate, but not by ether. The extraction is carried out at a pH of 3.2 to 3.4. The methyl ester melts, in various instances, from 258–262°. In Paper I (1) it was shown that this porphyrin, hitherto regarded as an entity and believed by Waldenström (2, 3) and others (4–11) to represent uroporphyrin III, is in many instances separable on a Tswett column ($CaCO_3$) into two porphyrins, the majority being uroporphyrin I methyl ester, m.p. 284°, the minority, a heptamethyl ester, m.p. 208°. Decarboxylation of the latter was productive, in part, of coproporphyrin III. Thus it was evident in these cases that the Waldenström porphyrin was a mixture of Type I and Type III porphyrins, but no evidence was obtained for the existence of a uroporphyrin III. The present study has concerned itself with porphyrins obtained according to Waldenström's method and whose methyl esters melted variously, from 258–272°, but which could not be separated into two porphyrins on the $CaCO_3$ column. These have already been alluded to in Paper I. The possibility existed that in these instances a uroporphyrin III was present, and the purpose of the study, therefore, was to determine the nature and isomer type of these variant porphyrins. We also desired to obtain further information as to whether the method of isolation affects the character of the porphyrin obtained. The results noted in Paper I had indicated that it was immaterial whether talc adsorption or ethyl acetate extraction was employed initially. Another purpose of the present study was to investigate the characteristics of artificial mixtures, in varying proportion, of the 208° porphyrin and the Waldenström porphyrin, both the type behaving as an entity on the Tswett column and that separable into 284° and 208° porphyrins being employed. The purpose here was to note whether such mixtures might be separated readily on the Tswett column, or whether there was any evidence of molecular compound formation.

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EXPERIMENTAL

Isolations

Urine samples were available from eight additional patients,[1] Cases 16 to 23, in our series. Further collections of feces were obtained from Cases 5 and 15, and a sample of feces from Case 23 (congenital porphyria) was also available for study. Autopsy was performed on Case 5 after the studies in Paper I had been completed; the liver and gallbladder bile were investigated in the present study. With the exception of Case 23, all of the cases were of the intermittent acute type. In Case 22 it was questionable whether any of the symptoms were related to the porphyria, the patient, a 64 year-old male, being admitted because of an acute thrombophlebitis, pulmonary infarction, together with transitory diarrhea and abdominal pain. Case 23[1] is an 18 month-old male infant suffering from congenital porphyria and exhibiting erythrodontia.

The methods and equipment were, in general, the same as described in Paper I. The porphobilinogen (3) reaction, which will be referred to as occurring in the native urine, has been described previously (12). Four types of procedures were used to isolate the porphyrins: (1) direct ethyl acetate extraction of the urine after addition of sufficient concentrated HCl to make a gray reaction to Congo red paper (pH 3.2 to 3.4); (2) talc adsorption of the urine after acidification with glacial acetic acid; (3) preliminary extraction with ethyl ether, after acidification with glacial acetic acid, to remove ether-soluble porphyrins and subsequent extraction with ethyl acetate after addition of sufficient concentrated HCl to bring the pH to 3.2 to 3.4; (4) preliminary extraction with ethyl ether, after acidification with glacial acetic acid, to remove ether-soluble porphyrins and subsequent talc adsorption. The application of the above procedures varied in certain respects, from sample to sample, as may be noted in the following.

Case 16—Porphobilinogen reaction, positive; zinc complex spectrum in the native urine with absorption maxima at 578 and 538 mμ. (*a*) 500 cc. of urine. Concentrated HCl was added to yield a gray reaction to Congo red paper. The urine was then extracted three times with 200 cc. amounts of ethyl acetate. The combined ethyl acetate was extracted four times with 10 cc. amounts of 10 per cent HCl. The combined HCl was then mixed with 3 volumes of methyl alcohol saturated in the cold with HCl gas. After standing overnight the total porphyrin ester was taken into chloroform and purified in the usual way (see Paper I), after which it was crystallized from chloroform-methyl alcohol. The melting points of the first crop of crystals and of serial recrystallizations were 248–252°, 256–260°, 261–262°, 261–263°. The latter crystals were further subjected to chromatographic analysis and decarboxylation, as noted in Table I, where they are designated as Case 16a. (*b*) 1000 cc. of urine were acidified with glacial acetic acid to pH 4.0. The total porphyrin was then adsorbed on talc and eluted with methyl alcohol-HCl as described in Paper I. The total porphyrin ester was taken into chloroform, purified in the usual way, and evaporated to dryness on the water bath; the residue was dissolved in benzene and mixed with one-half its volume of petroleum ether; chromatographic analysis with chloroform-benzene (1:20) as shown in Table I, Case 16b, was then carried out.

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[1] We are indebted to the following individuals for making available the urine samples and case analyses of six of the patients: Case 17, Dr. J. T. Wearn and Dr. John Gilson, Lakeside Hospital, Cleveland, Ohio; Cases 18 to 21, Dr. W. Halsey Barker and Dr. J. Shepheard, Johns Hopkins Hospital, Baltimore, Maryland; Case 23, Dr. James Orten, Department of Biochemistry, and Dr. Clement Smith and Dr. A. S. Nadas, Department of Pediatrics, Wayne University Medical School, Detroit, Michigan.

Case 17—300 cc. of urine. Porphobilinogen reaction, positive; zinc complex absorption was present in the native urine, as noted above. The urine was acidified with glacial acetic acid and then extracted repeatedly with ethyl ether to remove ether-soluble porphyrins. The ether fraction was not further worked up in this instance. The residual urine was acidified with concentrated HCl to a gray-blue reaction with Congo red paper, after which it was extracted repeatedly with ethyl acetate. The porphyrin was then removed from the latter by repeated extraction with small amounts of 10 per cent HCl after which it was esterified by addition of 3 volumes of methyl alcohol-HCl. The porphyrin ester was purified and crystallized from chloroform-methyl alcohol. M.p. 256°; after one recrystallization, 260–263°. Further disposal was as noted in Table I, Case 17.

Case 18—Porphobilinogen reaction, positive; zinc complex spectrum in the native urine, as given above. (*a*) 250 cc. of urine were acidified with glacial acetic acid and extracted repeatedly with ethyl ether to remove coproporphyrin. This fraction was not further worked up. The residual urine was acidified with concentrated HCl to a gray-blue reaction with Congo red paper, after which it was extracted repeatedly with ethyl acetate which was then further treated as described for Case 17. The crystalline porphyrin ester obtained melted at 250°. This compound, however, was lost prior to further study. The residual urine was subjected to talc adsorption, as described in Paper I. The porphyrin was eluted and esterified by methyl alcohol-HCl, taken into chloroform, purified in the usual way, and concentrated to dryness. The dry residue was dissolved in benzene-chloroform and chromatographed on $CaCO_3$. Five zones were noted, the next to the bottom of which was a porphyrin ester melting at 263°. The further treatment of this compound is indicated in Table I, Case 18a. The other zones were found not to be porphyrins. (*b*) 1800 cc. of urine were acidified with glacial acetic acid to pH 4.0 and the total porphyrins contained were adsorbed on talc and then eluted with methyl alcohol-HCl, after which the porphyrin ester was taken into chloroform, purified in the usual manner, and evaporated to dryness. This residue was chromatographed and the porphyrin zones were separated from other urinary pigments. The porphyrin fraction was further subjected to chromatographic analysis as shown in Table I, Case 18b. (*c*) 2400 cc. were acidified with sufficient concentrated HCl to give a gray reaction to Congo red paper. The urine was then extracted four times with ethyl acetate. In this instance only a small amount of porphyrin was obtained in the ethyl acetate; the larger fraction precipitated at the interphase. The porphyrin in the ethyl acetate was extracted with 10 per cent HCl, after which it was esterified, purified, and recrystallized four times to a constant melting point of 262–263°. The further treatment of this substance is shown in Table I, Case 18c. The porphyrin precipitate was also esterified and crystallized. After several recrystallizations, a constant melting point of 260–264° was obtained. The absorption spectrum was also identical with that observed in Case 18c. This compound was not studied further. (*d*) 500 cc. of urine were acidified with glacial acetic acid and extracted four times with ethyl ether to remove the coproporphyrin. This was taken into 10 per cent HCl, esterified with 3 volumes of methyl alcohol-HCl, and crystallized. It was found to consist mainly of coproporphyrin I, the methyl ester melting point being 240–242°. The residual urine was acidified with sufficient concentrated HCl to give a gray reaction to Congo red paper and was then extracted three times with ethyl acetate. The combined ethyl acetate was extracted four times with 10 per cent HCl and the porphyrin was esterified by addition of 3 volumes of methyl alcohol-HCl. The porphyrin ester was purified and crystallized in the usual manner. The crystals obtained, which melted at 258–259°, were subjected to chromatographic analysis, as noted in Table I, where they are designated as Case 18d. A very small amount of porphyrin remained in the urine after the ether and ethyl acetate extractions. This was adsorbed on talc after neutralization of the HCl with sodium acetate and addition of glacial acetic acid to pH 4.0. The porphyrin was eluted and esterified with methyl alcohol-HCl. The crystals obtained likewise melted at 261–262°, after having been rechromatographed twice and recrystallized repeatedly. The absorption spectrum was identical with that of the porphyrin from ethyl acetate.

Case 19—Porphobilinogen reaction, positive; zinc complex absorption in the native urine. (*a*) 500 cc. of urine were acidified with glacial acetic acid and extracted with ethyl ether to remove the coproporphyrin. HCl was added to the residual urine to give a gray reaction to Congo red paper, after which the porphyrin was extracted by ethyl acetate and was then taken from this into 10 per cent HCl. After being esterified and crystallized in the usual way, the ester obtained melted at 260–262°. It was further treated as shown in Table I, where it is designated as Case 19a. (*b*) 3000 cc. of urine were acidified with glacial acetic acid to pH 4.0. Talc adsorption followed by elution with methyl alcohol-HCl was then effected; the porphyrin methyl ester thus obtained melted at 252–256° and was further treated as indicated in Table I, Case 19b.

Case 20—2000 cc. Porphobilinogen reaction, positive. After acidification with glacial acetic acid the coproporphyrin was extracted into ethyl ether. The methyl ester of this porphyrin, obtained in the usual manner, was found to consist of isomer Type III, m.p. 140–142°. After addition of HCl to the residual urine, the entire ether-insoluble porphyrin was taken into ethyl acetate and thence into 10 per cent HCl. After esterification and purification in the usual way, the porphyrin ester obtained melted at 182–184° (first crop). The melting point after three recrystallizations was constant at 250–252°. Further disposal was as noted in Table I, Case 20.

Case 21—600 cc. Porphobilinogen reaction, positive; zinc complex spectrum in the native urine. The urine was handled by preliminary ether and subsequent ethyl acetate extraction, as in the preceding case. A Type I coproporphyrin was obtained in this instance, the methyl ester melting at 240–242°. After three recrystallizations and prior to chromatographic analysis, the ether-insoluble porphyrin methyl ester melted at 258–261°. The further treatment of this porphyrin is shown in Table I, Case 21.

Case 9—Porphobilinogen reaction, positive; zinc complex spectrum in the native urine. (*a*) 4000 cc. of urine were treated as in the two preceding cases. The ether-soluble porphyrin in this instance was Type III coproporphyrin exhibiting a dimorphic melting point of 143–146° and on cooling and remelting, 160°. The methyl ester isolated from the ethyl acetate by the usual procedure melted at 258–259° after three recrystallizations. Further disposal was as noted in Table I, Case 9a. (*b*) 3000 cc. of urine were acidified with glacial acetic acid to pH 4.0. Talc adsorption and elution with methyl alcohol-HCl were then carried out. The chloroform solution of the porphyrin ester was evaporated to dryness, and the residue dissolved in benzene was subjected to chromatographic analysis to remove other urinary pigments. Further chromatography and decarboxylation of the porphyrin ester were carried out as noted in Table I, Case 9b.

Case 5—3000 cc. Porphobilinogen reaction, positive; zinc complex absorption spectrum in the native urine. The urine was first acidified with glacial acetic acid and the coproporphyrin fraction removed. Serial recrystallizations of the copro ester and its mother liquor after chromatographic analysis revealed that both Type I and Type III coproporphyrins were present as follows:

1st crop.	160–180°	Mother liquors combined and concentrated; rosettes of prisms melting at 140–141°, after three recrystallizations; coproporphyrin III methyl ester
Recrystallization 1.	182–195°	
" 2.	198–220°	
" 3.	224–230°	
" 4.	242–246°	Coproporphyrin I methyl ester
" 5.	242–245°	
" 6.	242–245°	

About three-fourths of the ether-insoluble porphyrin was extracted by ethyl acetate. The methyl ester obtained from this by the usual method melted at 275°. This porphyrin was further treated as shown in Table I, Case 5. The porphyrin remaining in the residual urine was adsorbed on talc following addition of sodium acetate and glacial acetic acid to pH 4.0; it was then eluted and esterified with methyl alcohol-HCl; the ester thus obtained likewise melted at 274–275° after chromatographic analysis. The absorption spectra of these two porphyrins were identical.

Case 22—2000 cc. Porphobilinogen reaction, positive; zinc complex absorption spectrum in the native urine. The urine was acidified with glacial acetic acid and extracted first with ethyl ether, and next with ethyl acetate, which removed all of the porphyrin. Coproporphyrin III methyl ester was obtained from the ether, after fractionation and esterification, while the porphyrin from the ethyl acetate was further treated as shown in Table I, Case 22.

Case 23—550 cc. of urine. Negative porphobilinogen reaction. Absorption spectrum of native urine: I, 618–610, 614 mμ; II, 580–570, 575 mμ; III, 546–530, 538 mμ; IV, 510–490, 500 mμ. (It appeared that little or none was present as a metal complex.) Preliminary extraction with ether removed most of the coproporphyrin and small amounts of uroporphyrin. These were separated on the $CaCO_3$ column and proved to be copro- and uroporphyrin I methyl esters, melting respectively at 249–250°, and 284°. The residual urine was next extracted with ethyl acetate, which removed moderate amounts of uroporphyrin and a small remaining fraction of coproporphyrin. These were likewise shown to be Type I isomers. Finally the residual urine was subjected to talc adsorption, and the esters obtained from the talc were chromatographed on $CaCO_3$. But one porphyrin was obtained; *i.e.*, uroporphyrin I methyl ester, m.p. 283–284°. This is designated in Table I as Case 23.

The gallbladder bile from Case 5 measured 65 cc. It was diluted with 10 volumes of water, after which it was subjected to the method given in Paper I. The only porphyrin present was coproporphyrin I methyl ester, m.p. 248°. The entire liver from Case 5, weighing 1600 gm., was subjected to the isolation procedure as described in Paper I, up to and including the adsorption on talc. The porphyrin was extracted from the talc and esterified simultaneously by means of methyl alcohol-HCl, after which the ester was purified and crystallized in the usual way. The crystals were subjected to $CaCO_3$ chromatography. Two porphyrins were isolated, coproporphyrin I methyl ester melting at 240–242°, and uroporphyrin I methyl ester, m.p. 282–284°. The 208° porphyrin was not observed nor was coproporphyrin III, although it will be remembered that the latter porphyrin was present in the urine of this patient in considerable amount. The melting point of the coproporphyrin I methyl ester was, however, sufficiently low that the possibility of a small admixture of coproporphyrin III cannot be excluded.

Three samples of feces from Case 5, each weighing approximately 100 gm., one sample from Case 15, weighing 125 gm., and one from Case 23, weighing 400 gm., were examined. In the first two runs from Case 5 and in those from Cases 15 and 23, the feces were at once ground thoroughly with methyl alcohol-HCl. The mixture was allowed to stand overnight, after which it was filtered on a Büchner funnel. The porphyrin ester was taken into chloroform which was purified in the usual way, concentrated to dryness, and the residue chromatographed from benzene-petroleum ether, as given in Paper I. In the last run from Case 5, the feces were first ground repeatedly with ether which was discarded. The residue was then acidified with glacial acetic acid and the ether-soluble porphyrins were extracted by ethyl ether. The remaining feces were then ground with 10 per cent NH_4OH which was filtered off and acidified with glacial acetic acid. The porphyrins were then adsorbed on talc and eluted with methyl alcohol-HCl, after which they were further treated as described in Paper I. Each of the three runs from Case 5 yielded small amounts of coproporphyrin I methyl ester together with considerably larger amounts of a uroporphyrin methyl ester melting at 272–278°. In each instance the porphyrin behaved as an entity on the Tswett column, and the absorption maximum of the band in the red region was from 625.8 to 626 mμ. These findings indicate the presence of uroporphyrin I. The lower melting point is not explained (see Paper I).

The one run of feces from Case 15 yielded coproporphyrin III methyl ester, melting at 143–146°, together with a larger amount of uroporphyrin I methyl ester (m.p. 284°) and a porphyrin ester melting at 210°. The absorption spectra of the last two respectively were characteristic of the 284° and 208° porphyrins; *i.e.*, 625.8 and 625 mμ maxima for the bands in the red (in $CHCl_3$). The feces from Case 23 contained very large amounts of coproporphyrin I. Fluorophotometric measurement showed a concentration of approximately 65 mg. per 100 gm. By way of comparison it may be noted that the urine in this case contained but 0.5 mg. per 100 cc. of coproporphyrin, and 1.8 mg. per 100 cc. of uroporphyrin. The methyl ester melting point of the coproporphyrin from the feces was 249–250°. No evidence was obtained for the occurrence of uroporphyrin in the feces of this patient.[2]

Decarboxylations

These were carried out as described in Paper I. It was found to be immaterial whether the products of decarboxylation were esterified directly by addition of methyl alcohol-HCl, or after preliminary extraction with ether, and then from ether to dilute HCl, followed by esterification. A total of seventeen decarboxylations were carried out, eleven of which were primary in that they were done on the crude uroporphyrins isolated from the urines in Cases 5, 9b, 16a, 17, 18a, 18b, 18c, 19b, 20, 21, and 23. Secondary decarboxylations were carried out on porphyrins from the primary decarboxylations in Cases 9b, 18a, 18b, 18c, 19, and 21 (see Table I).

In Table I it may be noted that the coproporphyrins obtained in the secondary decarboxylations (Cases 18a, 18b, 18c, 19b, 21, and 9b) all exhibited methyl ester melting points between 236–246°, in all instances slightly lower than the melting point of pure coproporphyrin I methyl ester. It was essential to determine whether small amounts of coproporphyrin III were present, since if this were true, it would support the belief that the original 260° porphyrin, or the Waldenström porphyrin behaving as an entity, contained a small fraction of Type III isomer (the 208° porphyrin ?) bound in some way to a large fraction of uroporphyrin I. The individual amounts of coproporphyrin were too small in each instance to permit fractional crystallization of Type I and III isomers, but since the problem was the same in each case, the crystals from each were combined, rechromatographed, and recrystallized. The substance thus obtained melted at 248°; so that a greater degree of purity had been achieved. The mother liquor was further concentrated but, since no further crystallization occurred, the copper complex was prepared, as described in Paper I. This melted, after repeated recrystallizations, at 200° (coproporphyrin III methyl ester copper complex). The copper complex prepared from the coproporphyrin I methyl ester melting at 248° was noted to melt at 262°, as expected. From the copper complex of the coproporphyrin III methyl ester it was now possible to prepare the free ester. The copper was split out by treatment with concentrated sulfuric acid, after which the porphyrin was reesterified and chromatographed in the usual way. The resulting crystals from methyl alcohol melted at 140–142°, and the absorption was that of a coproporphyrin methyl ester. Thus definite evidence was obtained for the presence of a small amount of coproporphyrin III, with a large amount of coproporphyrin I, in the original mixture. In this connection it is quite probable that the native coproporphyrin I methyl ester isolated in various instances (see Table I) contained small fractions of coproporphyrin III, since the melting points were slightly low.

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[2] Since this was written, three additional samples of feces from this patient were made available to us. Uroporphyrin was not found in any, nor was there any ether-insoluble porphyrin. It is noteworthy, however, that while the first three of the four samples contained coproporphyrin I, the fourth sample received about 2 months later contained coproporphyrin III together with considerable protoporphyrin 9, and, again, a relatively large amount of coproporphyrin I, estimated at approximately 10 times that of the Type III isomer. The significance of the presence of the latter in this sample is not clear.

In Table I, it will be noted that the porphyrin esters obtained after chromatography of the primary decarboxylation products melted for the most part in the range indicating either the 224° porphyrin or coproporphyrin I. In Case 18a the melting point was lower, at 208–212°. This together with the higher absorption maxima indicated a mixture of the 208° and 224° porphyrins. Correspondingly, the coproporphyrin obtained by secondary decarboxylation melted at 236°. It may be that much of the coproporphyrin III described in the foregoing paragraph was donated from this case.

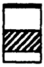
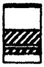
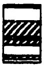
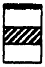
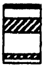
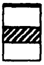
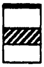
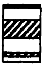
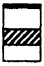
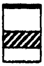
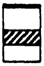
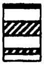
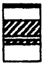
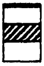
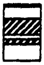
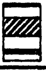
The decarboxylation of the 284° porphyrin from Case 23 was of particular interest with respect to the 224° porphyrin, since it was the only product. This proves that it is derived solely from the Type I isomer, since in this case there was no evidence for the presence of any of Type III.

Studies of Artificial Mixtures

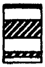
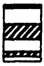
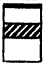
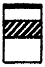
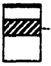
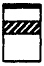
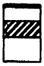
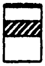
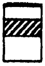
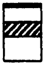
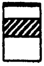
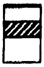
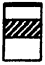
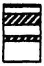
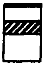
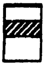
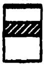
A series of experiments was carried out in which mixtures consisting of varying proportions of the 258–262° porphyrin and the 208° porphyrin were prepared The mixtures were studied as to melting point, chromatographic behavior, and decarboxylation products. These experiments are best described as follows:

A mixture was prepared consisting of porphyrin methyl esters from Case 21 and from Case 15 (approximately 3:1). The ester melting point of the former was 262° and that of the latter 258°. The former was an entity on the $CaCO_3$ column, while the latter was readily separated into 284° and 208° porphyrins, in confirmation of the result given for this material in Paper I. The mixed melting point was 257–258°. This mixture could not be resolved on a $CaCO_3$ column, exhibiting but a single zone. Decarboxylation was then carried out. Chromatographic analysis of the product yielded but a single zone. The absorption band in the red for this porphyrin dissolved in chloroform was 623.1 mμ. After three recrystallizations, the melting point was diffuse at 200–224°. The compound was rechromatographed and recrystallized with identical behavior, the melting point being 190–224°.

Table I Summary of characteristics of porphyrins

Case Number	Isomer type of copro-ester	Uro-type (ether insoluble) porphyrin esters isolated		Primary chromatogram ($CaCO_3$)	Porphyrin esters obtained from primary chromatogram	
		M.P. in °C.	Abs. Max.* in $CHCl_3$ in mμ		M.P. in °C.	Abs. Max.* in $CHCl_3$ in mμ
5	I & III M.P. 242-45° M.P. 140-41°	265	625.7		275-76	625.6 →
9 a	III M.P. 143-46°	258-59	625.5		280-84 207-08	626.2 625.0
9 b	III M.P. 142-43°	dry residue	625.5		284 208 142-43	625.7 } 624.8 } → 622.1
16 a	not exam.	261-63	625.6		261-62	625.5 →
16 b	not exam.	dry residue	624.6		284	625.9 622.1
17	not exam.	260-63	625.6		263-64	625.5 →
18 a	not exam.	262	625.8		263	625.6 →
18 b	not exam.	dry residue	625.0		263-65	625.7 → 622.1
18 c	not exam.	260-62	625.7		264	625.6 →
18 d	I M.P. 240-42°	258-59	625.9		260-62	625.7
19 a	not exam.	260-62	625.8		260-63	625.6
19 b	I M.P. 240-41°	dry residue	624.6		260-62 240-41	625.2 → 621.1
20	III M.P. 142-44°	250-52	625.0		252-54 206-09	625.7 → 624.9
21	I M.P. 240-42°	258-61	625.7		260-61	625.6 →
22	III M.P. 142-43°	258-59	625.2		282-84 207-08	625.7 624.8
23	I M.P. 249-50°	dry residue	625.8		282-84 250	625.8 → 621.1

isolated from urines of various porphyria cases

Porphyrin esters obtained after primary decarboxylation			Porphyrin esters obtained after second decarboxylation		
Chromatogram ($CaCO_3$)	M.P. in °C.	Abs. Max.* in $CHCl_3$ in mμ	Chromatogram ($CaCO_3$)	M.P. in °C.	Abs. Max.* in $CHCl_3$ in mμ
→ 	221-24 245-48	623.8 622.1			
→ 	221-24 142-44	623.8 621.3	→ 	239-42	621.8
→ 	244-46	622.2			
→ 	244-48	622.2			
→ 	208-12	624.7	→ 	236	622.2
→ 	224	623.1	→ 	240-41	622.1
→ 	224-26	623.3	→ 	238-40	622.4
→ 	219-26	623.5	→ 	238-40	622.1
→ 	219-21 240-42	623.4 622.1			
→ 	224	623.1	→ 	242-46	622.2
→ 	223-24	623.4			

Note: methyl esters are referred to in all instances

* Redward band

The material was again decarboxylated and the products were chromatographed. The band was now 622.1 mμ (in $CHCl_3$). After three recrystallizations the melting point was 228–232°. In view of the fact that but one zone was present (on the chromatogram) this melting point was believed to represent a mixture of relatively much coproporphyrin I and relatively little coproporphyrin III, as expected from the original mixture.

A mixture was prepared consisting of 3 parts of the porphyrin methyl ester melting at 267–271° from Case 5 and 1 part of the 208° porphyrin from Case 9. Both of these behaved as entities on the $CaCO_3$ column. Chromatographic analysis of the mixture yielded two zones with melting points of 260° and 208°. The crystals from each of these zones were mixed and decarboxylated together. The crystals of the methyl ester thus obtained resembled those of coproporphyrin III and exhibited a constant melting point at 160–161° after four recrystallizations. These crystals and the mother liquor, which was very red, were mixed and chromatographed together, yielding two zones on the column, the first of which exhibited an absorption band in the red of 622.8 mμ, and a melting point of 221–224° after three crystallizations; the second, an absorption spectrum of 622 mμ and a melting point of 142–144° after four crystallizations. The crystals were characteristic or coproporphyrin III methyl ester. The first zone was further decarboxylated, crystallized, chromatographed, and recrystallized three times to give an ester with an absorption band having a maximum of 621.8 mμ, and a melting point of 240–242°. These characteristics and the type of crystal served to identify the substance as the methyl ester of coproporphyrin I.

A mixture of porphyrin methyl esters was prepared consisting of 1 part of the 262° porphyrin from Case 18 and 3 parts of the 258° porphyrin from Case 9. The former ester was an entity on the $CaCO_3$ column, while the latter was easily separated into the 284° and 208° porphyrins. This mixture yielded two zones on chromatographic analysis, the first zone melting at 268–269° and the second at 207–208°. Decarboxylation of the mixture of these two zones and subsequent chromatographic analysis of the product yielded one zone with an absorption spectrum of 622 mμ and the following melting points, on serial recrystallization: (1) 178–186°, (2) 198–208°, (3) 216–224°, (4) 224–230°, (5) 230–236°, (6) 238–240°, (7) 236–240°.

The mother liquor from the first three recrystallizations was combined and further concentrated, yielding rosettes of prisms melting at 140–142° (coproporphyrin III methyl ester).

A mixture of porphyrin esters was prepared consisting of 4 parts of a chromatographic entity melting at 262° from Case 18 and 1 part of the 208° porphyrin from Case 9. This mixture behaved as one porphyrin on the $CaCO_3$ column, and the melting point was 257–260°. Upon decarboxylation and chromatography but a single zone was obtained. This exhibited an absorption band in the red having a maximum of 622.8 mμ. The melting point was 231–234° after five recrystallizations. The mother liquor was somewhat colored but failed to yield additional crystals on further concentration.

On many occasions mixtures of the pure 284° and 208° porphyrin esters from various sources have been resolved into their two characteristic zones on the Tswett column, and at no time has any evidence been noted of a combination of these porphyrins following artificial mixture. On the other hand, mixtures of the 284° and the 260° porphyrins (or Type B Waldenström porphyrin, see the diagram below), regardless of proportion, behave as entities on the Tswett column ($CaCO_3$).

Thus, three mixtures of the 260° and 284° porphyrins, in varying proportion, were dissolved and chromatographed. In each instance but one zone resulted. The proportions of the mixture, and the melting points of the crystals from the resultant zones, were as follows:

3 parts of 260° + 1 part of 284° → 268–269°
1 part " 260° + 1 " " 284° → 268–270°
1 " " 260° + 3 parts " 284° → 270–272°

This rather surprising result makes it clear that melting points lower than 284°, as discussed in Paper I, may be due in some instances at least to the presence of a small amount of the 260° porphyrin, quite possibly in molecular combination with uroporphyrin I.

Solubilities

It was pointed out in Paper I that the 208° porphyrin, once purified, could be saponified and extracted quantitatively by ethyl acetate from the aqueous phase after the pH is adjusted to 3.2 to 3.4. The pure 284° porphyrin is not extracted by ethyl acetate. This has been confirmed in the present study for the 284° porphyrin isolated from the liver of Case 5. It may be noted, however, that about one-third of the 284° porphyrin in the urine of Case 23 (congenital porphyria) was extracted by ethyl acetate. This can only be assumed to have been conditioned by impurities in the urine, since after saponification of the crystalline ester the free uroporphyrin was quite insoluble in ethyl acetate and was not extracted from an aqueous solution of pH 3 2 to 3.4.

Porphyrins of the Waldenstrom type isolated from Cases 5, 9a, 17, and 18c, and whose methyl esters melted variously between 258–275°, were saponified and brought into a dilute HCl solution of pH 3.2 to 3.4. In every instance the porphyrin was extracted quantitatively from these solutions by ethyl acetate.

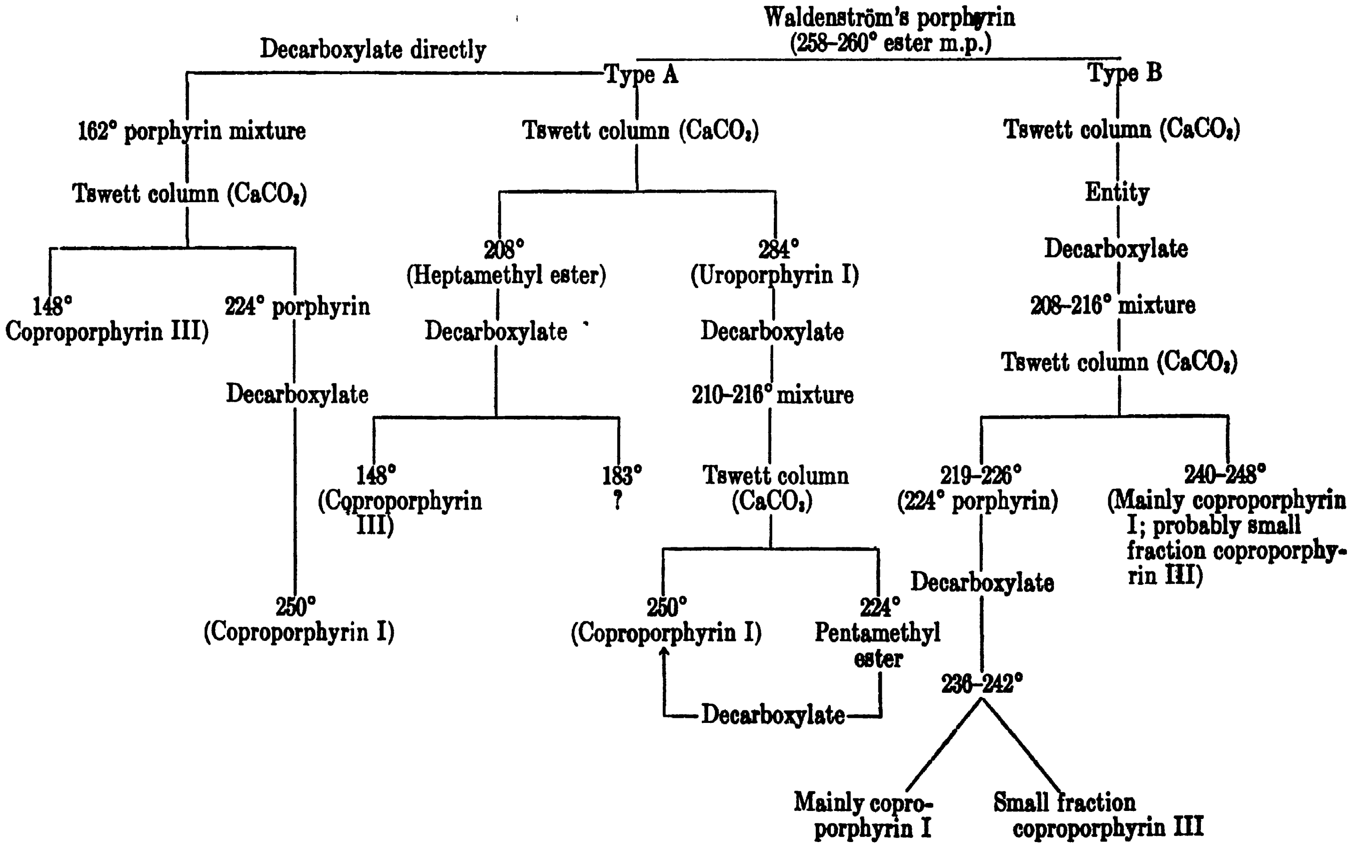
DISCUSSION

The present study has made it clear that the Waldenström type porphyrin as isolated from the urine of certain porphyria cases behaves as an entity, while in other instances it is readily separable into uroporphyrin I and the 208° porphyrin, the latter, according to the results in Paper I, having but seven carboxyl groups. The porphyrin behaving as an entity on the Tswett column appears to possess a limited binding power for the 208° porphyrin, as shown in the mixture experiments. This quality is not possessed by the pure 284° porphyrin (uroporphyrin I methyl ester), since mixtures of it with 208° porphyrin are readily resolved on the Tswett column. The binding of 208° porphyrin by the Waldenström porphyrin is distinctly limited as to quantity; if larger amounts are added, they may be separated again on the calcium carbonate column. The fact that this binding power exists suggests that that type of Waldenström porphyrin which behaves as an entity may represent a chemical combination of the 284° and 208° porphyrins. The possibility must also be considered that the Waldenström porphyrin in these instances represents a molecular combination of other, as yet unidentified, Type I and Type III porphyrin isomers. The decarboxylation studies are quite in harmony with either possibility, since they show that the initial 260° porphyrin consisted of a large fraction of Type I and a small fraction of Type III isomers. This is clearly revealed in the secondary decarboxylations in which a mixture of much coproporphyrin I and little coproporphyrin III was proved to be present.

The present studies, together with those of Paper I, permit delineation of the relationships shown in the accompanying diagram. This diagram is self-explanatory with one exception. It is noted that direct decarboxylation of the Type A Waldenström porphyrin yields the porphyrin mixture melting at 162°, while decarboxylation of the Type B porphyrin is productive of a mixture melting at 208–216°. Coproporphyrin III is easily obtained from the former, but as noted above, only with difficulty and in very small amount from the latter. This difference is believed to indicate that the Type B Waldenström porphyrin contains much less of isomer Type III than is true of the Type A.

These studies have also permitted a better comparison of the urines in the congenital and intermittent acute types of porphyria. This is based, however, on but two cases of the congenital type as contrasted with twenty-one of the intermittent acute variety. The differences are given in Table II. One difference not included in this table is the absence of uroporphyrin in the feces of congenital porphyria. This, however, has been ascertained for but one case, so that further studies are required.

The results of the present study thus make it quite clear that the uroporphyrin in all cases of porphyria is composed at least mainly of porphyrin


Waldenström's porphyrin
(258–260° ester m.p.)
Decarboxylate directly
Type A
Type B
162° porphyrin mixture
Tswett column (CaCO₃)
148° Coproporphyrin III)
224° porphyrin
Decarboxylate
250° (Coproporphyrin I)
Tswett column (CaCO₃)
208° (Heptamethyl ester)
Decarboxylate
148° (Coproporphyrin III)
183° ?
284° (Uroporphyrin I)
Decarboxylate
210–216° mixture
Tswett column (CaCO₃)
250° (Coproporphyrin I)
224° Pentamethyl ester
Decarboxylate
Tswett column (CaCO₃)
Entity
Decarboxylate
208–216° mixture
Tswett column (CaCO₃)
219–226° (224° porphyrin)
Decarboxylate
236–242°
Mainly coproporphyrin I
Small fraction coproporphyrin III
240–248° (Mainly coproporphyrin I; probably small fraction coproporphyrin III)

corresponding in configuration to etioporphyrin I. It appears that the Type I isomer alone is excreted in congenital porphyria; while in the intermittent acute type the situation is more complicated, the Type I isomer predominating, but in association with minor fractions of porphyrin corresponding to etioporphyrin III. This association is represented in some cases by a mixture of uroporphyrin I with the 208° porphyrin, a heptamethyl ester of Type III configuration; in others by a combination of relatively much Type I and relatively little Type III porphyrin, which may be, respectively, the 284° and 208° porphyrins, although this has not been proved. Whether occurring as a mixture separable on the Tswett column, or as a molecular combination behaving as an entity, the porphyrin initially obtained from the urine by Waldenström's ethyl acetate extraction, or by other methods, exhibits an ester melting point in the neighborhood of 260°. This porphyrin is insoluble in ether and thus resembles uroporphyrin I, but is not its isomer, nor has any evidence been obtained in the present study for the occurrence in nature of a uroporphyrin III.

TABLE II

Characteristic Differences in Urines of Congenital and Intermittent Acute Types of Porphyria

Congenital	Intermittent acute
Majority or all of porphyrin in free state, not combined with zinc	All or most of porphyrin present as zinc complex
Relatively large amounts of uro- and coproporphyrin I	Small amounts of porphyrin consisting of mixture of 260° porphyrin with coproporphyrin, either Type I or III, or a mixture
Porphobilinogen absent	Much porphobilinogen and porphobilin
Urine purer red in color	Urine exhibits browner tint

The variation in excretion of the coproporphyrin isomers is not understood. In some instances only coproporphyrin I was obtained, in others only coproporphyrin III, and in some instances a mixture of the isomers. This variation bore no obvious relationship to other factors.

SUMMARY

1. A study of the urine porphyrins from eight additional instances of porphyria, seven of the intermittent acute, and one of the congenital type, has failed to yield any evidence for the occurrence of uroporphyrin III. In five of the seven cases of the intermittent acute type the Waldenström porphyrin behaved as a chromatographic entity. Evidence is presented to show that this porphyrin is a combination of a large amount of Type I

and a small amount of Type III isomers. In two of the seven cases the Waldenström porphyrin as initially isolated behaved as described in Paper I, being readily separated chromatographically into the 284° and 208° porphyrins. In the case of congenital porphyria only Type I isomer was present, the urine containing uroporphyrin I and coproporphyrin I in an approximate ratio of 3:1.

2. The feces from two intermittent acute cases contained uroporphyrin I, in one instance associated with coproporphyrin I, and in the other with considerable coproporphyrin III, together with the 208° porphyrin. The significance of this variation is not clear. The feces from the congenital porphyria case contained coproporphyrin I, alone, in large amount in three of four runs, while in the fourth, a small fraction of coproporphyrin III was also present. Uroporphyrin was not found in any of the four samples.

3. Uro- and coproporphyrin I were isolated from the liver of one of the intermittent acute cases dying of the disease. The gallbladder bile in this case contained only coproporphyrin I.

4. The method of isolation was found not to be material with respect to the character of the porphyrins of either urine, feces, or liver.

BIBLIOGRAPHY

1. Grinstein, M , Schwartz, S , and Watson, C J , *J Biol Chem.*, **157,** 323 (1945)
2 Waldenstrom, J , *Deut Arch klin Med* , **178,** 38 (1935), *Acta med Scand* , suppl (1937)
3 Waldenstrom, J , and Vahlquist, B , *Z physiol Chem* , **260,** 190 (1939).
4 Fischer, H , and Hofmann, H , *Z physiol. Chem* , **246,** 15 (1936)
5. Mertens, E , *Z physiol Chem.*, **250,** 57 (1937)
6 Turner, W. J , *J Lab and Clin Med.*, **26,** 323 (1940)
7. Rimington, C , *Compt -rend. trav. Lab. Carlsberg, Série chim.*, **22,** 454 (1938).
8. Chandler, F S., Harrison, S. A., and Rimington, C., *Brit. Med. J.*, 1173 (1939).
9. Dobriner, K , and Rhoads, C. P , *Physiol. Rev* , **20,** 416 (1940).
10. Watson, C. J , *South. Med J.*, **36,** 359 (1943)
11. Nesbitt, S., *J. Am Med. Assn.*, **124,** 286 (1944).
12. Watson, C J , and Schwartz, S , *Proc Soc Exp. Biol. and Med.*, **47,** 393 (1941).

THE RELATIONSHIP OF THE ADENOSINE POLYPHOSPHATES TO FATTY ACID OXIDATION IN HOMOGENIZED LIVER PREPARATIONS*

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Perhaps the greatest single difficulty encountered in the study of the intimate mechanisms involved in the oxidation of fatty acids to smaller fragments has been the fact, noted by Quastel and Wheatley (1), that when liver cells are ruptured or disorganized *in vitro* they lose the power to oxidize fatty acids. This observation has been repeated often and it is striking, because this oxidation is one of the most prominent metabolic activities of the liver *in vivo*. As a consequence of this property, the study of the enzymes and mechanisms of the oxidation has been confined to perfusion and surviving slice techniques, which have obvious limitations. Since the use of minced tissue and tissue extracts has enabled investigators to make detailed deductions about the mechanism of glycolysis and the terminal oxidation of pyruvic acid, it is felt that comparable progress cannot be made with reference to fatty acid oxidation until such oxidation can also be realized in minced liver or in liver extracts.

In 1939, Leloir and Muñoz (2) reported a limited success in this direction. They found that when liver was homogenized at low temperatures it sometimes retained the power to oxidize butyric acid, as evidenced by production of the ketone bodies. It was found necessary to pass oxygen through the brei at all times to maintain its activity. The 4-carbon dicarboxylic acids enhanced this activity. These observations have been confirmed in this laboratory. The effect is not always obtained and even an active preparation retains its activity for only a period of minutes. These data were extended by the same authors in 1943 (3) when they described fatty acid oxidation by a partially purified enzyme preparation from guinea pig liver. This enzyme, in the presence of adenylic acid, inorganic phosphate, magnesium ions, cytochrome, and a high concentration of fumarate was able to oxidize the lower fatty acids (up to 8 carbon atoms) at rates which were not always reproducible in different preparations. The preparations were not always successful and were stable for only short periods. Other significant features, also confirmed by us, were the facts that a boiled tissue juice could sometimes activate the system and that determinations of phosphorus exchanges in the reaction medium showed that there was a decrease in adenosine triphosphate (ATP) phosphorus and phosphopyruvic acid P and an increase in inorganic phosphate in the presence of butyric acid. Leloir and Muñoz felt these changes indicated that the oxidation of the fatty acid was in some way coupled with a phosphorylation but they did not suggest a possible mechanism for this effect.

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* The substance of this communication was presented in preliminary form as a "Letter to the Editors" of this *Journal* in June, 1944 (*J. Biol. Chem.*, **154**, 309 (1944)).

As a consequence of many observations made in this laboratory since 1940 and the data reported by Leloir and Muñoz in their pioneering attack on this difficult problem, it was found possible to explain these facts satisfactorily and prepare liver homogenates, stable for relatively long periods, which would consistently oxidize fatty acids having up to 18 carbon atoms at a high and fairly reproducible rate. These preparations offer a direct and practical approach to the detailed study of the oxidation of higher fatty acids.

The difficulty in obtaining active liver minces and extracts without certain special precautions and treatments as described by Leloir and Muñoz can be explained if it is assumed that a relatively high concentration of adenosine triphosphate (or the diphosphate) is required to activate or in some manner facilitate the oxidation of the fatty acid by the liver preparation. Reasons for this assumption are enumerated.

1. ATP is known to be rapidly dephosphorylated on stimulation or disruption of cell structure, by specific or non-specific phosphatases (4). In the preparation of a liver extract, dilution of cell constituents reduces the ATP concentration still further. Any broken cell preparation used for testing fatty acid oxidation would be very low or lacking in ATP.

2. The work of Kalckar (5), Warburg and Christian (6), Colowick and associates (7), Ochoa (8), and others has amply demonstrated that with the oxidation of carbohydrate material in tissue extracts inorganic phosphate is esterified and adenylic acid becomes phoshorylated to the energy-rich di- and triphosphates. The essential components of such a system, namely glucose (or the C_4 acids, pyruvate, etc.), adenylic acid, inorganic phosphate, and the usual hydrogen transport factors, are exactly those which were necessary to support fatty acid oxidation in the experiments of Muñoz and Leloir. The fumarate they used provided substrate for oxidations which were capable of phosphorylating adenylic acid to ATP, which in turn is presumed necessary for the oxidation of fatty acids in such systems.

3. The necessity for oxygenation of the liver brei earlier reported by Leloir and Muñoz is a corollary of (2). Oxygen is required to maintain the oxidations and hence the aerobic phosphorylations.

4. The activation by boiled tissue juice could be due to its content of ATP, although this would depend on its mode of preparation.

5. The figures presented by Muñoz and Leloir on the phosphorus distribution might well indicate that ATP was used up during fatty acid oxidation (decrease of easily hydrolyzed P compared to control extract) as was the phosphopyruvic acid, which is known to phosphorylate adenylic acid.

This interpretation of the data warranted a direct experimental trial of ATP in the activation of fatty acid oxidation. Muñoz and Leloir mentioned that ATP could be substituted for adenylic acid in their *complete* system, but in this experiment they could not have distinguished between a possible *specific* effect of ATP or its effect due to the formation of adenylic acid by dephosphorylation.

Preliminary experiments to test this interpretation showed that ATP could indeed activate the oxidation of fatty acids by a homogenized liver preparation and, after a study of optimum conditions for this activation was made, the activity and properties of a standard system were investigated, as is described in detail in the following sections.

EXPERIMENTAL

1. Preparative—The fatty acids used were pure Eastman products. The acids having up to 8 carbon atoms were redistilled; the higher acids were used as purchased. They were neutralized with the required amount of NaOH and adjusted before the experiment to pH 7.5. The acids having 10 or more carbon atoms were brought into solution at 60–70° with NaOH, adjusted to pH 8.0, and cooled. The higher acids exist partly as soaps and partly as free acids at this pH and not all the material is in solution at 25° in the stock concentrations used.

ATP was isolated from rabbit muscle following magnesium anesthesia (9) according to the method of Needham (10). It was usually carried through two precipitations of the mercury salt to give preparations having an acid-labile P to total P ratio of not less than 0.64.

Adenosine diphosphate (ADP) was prepared enzymatically from ATP by the method of Bailey (11). Purified myosin from rabbit muscle (11) was used as the specific phosphatase. The product had an acid-labile P to total P ratio of 0.48.

Inosine triphosphate was prepared by deamination of ATP with nitrous acid and inosine diphosphate was prepared enzymatically from the triphosphate according to Kleinzeller (12). They were of the same order of purity as the ATP and ADP. Muscle adenylic acid was a recrystallized commercial product (Laokoon). Phosphocreatine was prepared synthetically (13) and obtained analytically pure by alcohol precipitation of the calcium salt according to Fiske and Subbarow (14). Phosphopyruvic acid was a synthetic preparation (15) containing 19 per cent of its phosphorus as inorganic phosphate. Diphosphopyridine nucleotide was isolated from bakers' yeast by the method of Williamson and Green (16) and was 46 per cent pure, as determined spectrophotometrically after hydrosulfite reduction. Cytochrome *c* was prepared from beef heart by the method of Keilin and Hartree (17). Its concentration was also determined spectrophotometrically. Inosinic acid was isolated from rabbit muscle by the method of Ostern (18). The 3-phosphoglyceric acid and fructose-6-phosphate were gifts of Dr. G. A. LePage. Fructose diphosphate, adenosine, adenine, hypoxanthine, choline, thiamine pyrophosphate, and the glycerophosphates were commercial preparations.

In general, the degree of purity of all compounds used was established by appropriate analytical methods. The organic phosphates were converted into their sodium salts in suitable stock concentration and adjusted to pH 7.5 before use.

2. Analytical Methods—For measurements of oxygen uptake standard Warburg flasks of 16 ml. total volume were used. The experiments were run at 25° and in all cases the gas phase was air. The equilibration period was 5 minutes, at which time the taps were closed and the substrates tipped in from the side arm. Inorganic phosphate and the acid-labile phosphorus of the adenosine polyphosphates (liberated by 7 minutes hydrolysis at 100° in 1 N HCl) were determined on trichloroacetic acid filtrates of the reaction media by the method of Fiske and Subbarow (19), adapted to photoelectric measurement. The fatty acid determinations were performed by the distillation technique described by Leloir and Muñoz (2).

3. Preparation of Liver Homogenate—Adult white rats on a standard laboratory diet were decapitated and bled. The liver was removed, washed in a stream of distilled water, and chilled on crushed ice. It was then cut into small pieces with scissors and homogenized in the device of Potter and Elvehjem (20) with 2 volumes of ice-cold buffer. The buffer used was the Krebs-Ringer phosphate buffer (21) adjusted to pH 8.0. Calcium was omitted from the buffer. The preparation was then filtered through two layers of gauze and stored at 0° until ready for use. Prolonged homogenization in high speed, close fitting homogenizers was found to result in complete inactivation of the enzymes responsible for fatty acid oxidation without affecting the endogenous respiration. The optimum time for homogenization was 1 to 2 minutes for our apparatus; this factor was easily controlled by a pilot experiment on the particular apparatus used. Microscopic examination of the homogenate showed practically complete cell rupture. The homogenates were aged before use, as indicated for certain experiments, to allow maximum destruction of organic phosphates by phosphatases. All experiments reported in this paper were performed in duplicate on preparations from at least three rats and the data represent average figures.

4. Requirement of ATP for Oxidation of Octanoic Acid—To demonstrate the activating effect of ATP on octanoic acid oxidation the following system was found to give optimum results. The main compartment of the Warburg vessel contained 0.25 ml. of fresh homogenate (added just before equilibration), 0.20 ml. of 0.13 M phosphate buffer of pH 8.0, 0.10 ml. of cytochrome solution, and 0.25 ml. of ATP solution. The center well was equipped with a filter paper roll and 0.2 ml. of 10 per cent KOH, and the side arm contained 0.20 ml. of the sodium octanoate solution. After equilibration the substrate was tipped in and measurement of oxygen uptake begun. The flask then contained 0.8 ml. of reaction medium with a final concentration of 0.0025 M ATP, 4.0×10^{-5} M cytochrome, 0.0075 M sodium octanoate, 0.030 M disodium phosphate, 0.021 M sodium chloride, 0.0008 M potassium chloride, 0.0002 M magnesium sulfate, and approximately 85 mg. of homogenized liver tissue. The control flask (for determining the endogenous activity) contained 0.20 ml. of H_2O instead of the substrate solution. When components were omitted, a corresponding volume of water was substituted. The essential data of a typical experiment, demonstrating the requirement of ATP for oxidation, are shown in Fig. 1.

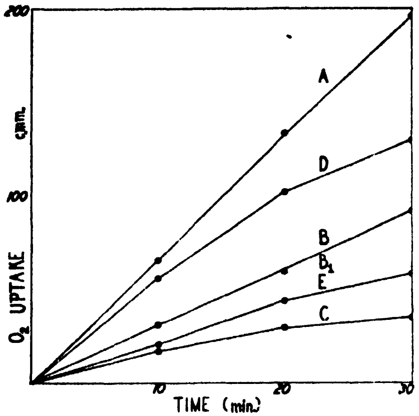

FIG 1 The requirement of adenosine triphosphate (ATP) in the oxidation of octanoate by rat liver homogenate Curve A, complete system + substrate + ATP; Curve B, complete system without substrate + ATP; Curve B_1, complete system without substrate or ATP; Curve C, complete system + substrate without ATP; Curve D, complete system + substrate + ATP without cytochrome *c*; Curve E, complete system without substrate + ATP without cytochrome *c*.

It will be seen that the presence of ATP allows a considerable extra oxygen uptake above the endogenous level. The necessity for the presence of ATP is shown by the curves representing the activity without ATP, in which case the endogenous level is almost identical with that in the presence of ATP, but in the presence of added octanoate there is an actual inhibition of respiration. This is probably due to generalized inhibition of enzymes by the surface activity of higher fatty acids and soaps. This inhibition has been noted before by Muñoz and Leloir in this connection and inhibition of other enzymes by fatty acids has been noted (for instance, several oxidases in liver extracts (22) and diastase (23)). This effect is covered more fully in a later section. The necessity of adding extra cytochrome to maintain the maximum total respiration and linearity of uptake is also evident.

The respiration falls off quickly after 40 minutes in this experiment because of the decrease in ATP concentration. If more ATP and substrate are added at this point from a second side arm, the respiration becomes linear again for 30 to 40 minutes. The difficulties involved in using higher substrate concentrations are considered in a later section.

That the extra oxygen uptake actually reflected oxidative destruction of the fatty acid was shown by determining the amount of octanoate before and after oxidation with and without ATP in a larger scale experiment (see Table I). The distillation and titration of small amounts of octanoic acid are not very accurate (20 per cent negative error) and the data are not considered to be quantitatively significant. They do show utilization of a great part of the octanoate in the presence of ATP, but none in its absence. The products of the reaction have not as yet been studied thoroughly but preliminary experiments showed the formation of approximately 1.2 moles of total ketones from each mole of octanoate in this experiment.

TABLE I

Oxidative Utilization of Octanoate

The experiment was arranged exactly as that shown in Fig 1, with all amounts multiplied by 6 to give a total volume of 6.0 ml. This was necessary to allow octanoate determination. Time, 30 minutes.

Adenosine triphosphate	Substrate	Oxygen uptake	Octanoate removed
		micromoles	*micromoles*
+	−	25 1	−1.2
+	+	52.5	24.3
−	−	26.3	−1.2
−	+	12.7	−1.3

5. Properties of System—When the enzyme system is prepared as described, with the necessary additions and the precaution of determining the optimum homogenization time, the experiment has been found to be quite reproducible in liver preparations from nearly 100 rats. There were no failures to obtain oxidation. The liver homogenate is stable and can be activated by the addition of ATP with no great change in activity 4 to 6 hours after preparation. Most preparations tested showed some activity even after storage at 0° for 18 hours and one preparation showed activity 3 days after the homogenization. It was found that the omission of either potassium or magnesium from the Krebs buffer did not affect appreciably the extra uptake due to fatty acid but did lower the total respiration. The same effect, but to a more pronounced degree, was found when both Mg^{++} and K^{+} were omitted.

The optimum pH for the reaction described lies between 7.0 and 8.0, the activity dropping off sharply outside this range. The question is complicated by the fact that below pH 7.0 the great surface activity of octanoic acid increases, inhibiting the reaction and also the residual respiration greatly.

Barbiturate, dimethylglycine, and borate buffers inhibited the oxidation. Although phosphate mixtures have little buffering power at pH 8.0, the reaction medium as described was the most active.

The use of more dilute liver homogenates resulted in decreased total respiration and a greater proportional decrease in endogenous activity. However, dilution beyond 25 mg. of tissue (wet weight) per 1.0 ml. of reaction medium greatly impaired the extra uptake due to fatty acid.

Even short periods of dialysis completely inactivated the homogenate so that it could not be reactivated by addition of ATP. Addition of ATP, diphosphopyridine nucleotide, K^{+}, Mg^{++}, cytochrome, and riboflavin restored a small part of the activity. Obviously other dialyzable components were necessary for complete restoration

When the effect of octanoate concentration on the rate of the oxidation was studied (ATP concentration, 0.00125 M), it was found that the rate increased steadily on raising the concentration of substrate from 0.0001 M to 0.0025 M, at which concentration the enzyme system seemed saturated. When the concentration of substrate was increased stepwise to 0.02 M, the rate fell off sharply and ATP activation was no longer noticeable at the latter concentration. This behavior is not typical of the ideal enzyme and it is probably a reflection of the surface activity of the higher fatty acids and soaps. This behavior was also shown in the optimum pH curve, since free octanoic acid is more surface-active than the sodium salt (24).

That the activation by ATP is peculiar to the fatty acid oxidation in the liver was shown by assay of different rat tissues with octanoate as substrate. The kidney was the only other organ showing any activation by ATP, but this tissue possessed less than a tenth of the activity of the liver. Muscle, brain, and intestine suspensions showed no activity under the same conditions.

6. Dependence of Rate of Octanoate Oxidation on ATP Concentration—When the ATP concentration was varied in the system described in section

(4), fresh homogenate being used in the presence of 0.007 M octanoate, the rate of oxidation varied with the concentration of ATP. The endogenous respiration was virtually unchanged in the presence of different amounts of ATP (see Fig. 2).

It can be seen that a minimum concentration of 1 × 10^{-4} M ATP is required to show detectable extra uptake in this experiment. Addition of ATP in concentrations higher than 0.0025 M did not result in proportional increases in rate; there was a tendency for the nucleotide to precipitate some of the tissue constituents gradually, forming slimy, ropy deposits.

It can be concluded that there is a real relationship between ATP concentration and the rate of fatty acid oxidation in these preparations. The concentration of adenosine polyphosphates in intact liver tissue of the rat, as determined by analysis of the organ instantaneously frozen *in situ* (to avoid hydrolysis on stimulation of cells), ranges between 0.001 and 0.002 M, which is comparable to the optimum range of ATP in these *in vitro* experiments (see also (25, 26)).

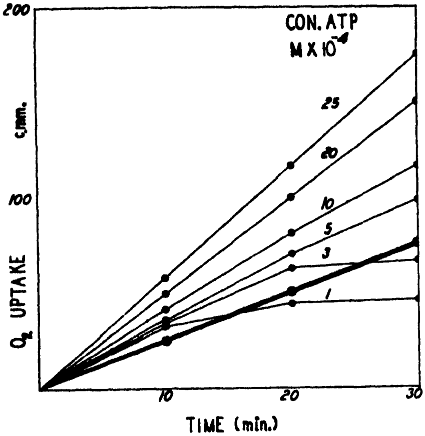

FIG. 2. The effect of the adenosine triphosphate (ATP) concentration on the rate of octanoate oxidation. The heavy line indicates the rate of the endogenous respiration (without substrate, but with ATP) and the lighter lines indicate the rate of oxidation in the presence of 0.007 M octanoate with variations in ATP concentration indicated.

It is also apparent that low concentrations of ATP activate the oxidation for only a short period; in the case of the higher concentrations of ATP the rate eventually falls after longer periods but can be restored by adding more ATP to the system.

7. *Specificity of ATP in Activating the Oxidation*—To determine whether the activation by ATP is actually due to its intact structure, to its meta-

bolic products, or whether this activation is a non-specific effect of phosphoric esters in general, the basic experiment of section (4) was repeated, various compounds being substituted for the ATP. In these experiments the final concentration of octanoate was 0.007 M. In every case an endogenous control (without octanoate) containing the compound assayed in equal concentration was run. The basis for the assay was the total extra O_2 uptake due to octanoate oxidation (above the endogenous uptake in the presence of the phosphorylated compound) at 50 minutes. An arbitrary value of 100 was set on the activation by ATP in 0.0025 M concentration, which served as a control in all experiments.

TABLE II

Effect of Different Compounds in Activating Octanoate Oxidation

The experimental details are given in section (7).

Compound	Concentration	Activation (ATP = 100)
	M	
Adenosine triphosphate	0.0025	100
" "	0.00125	70
" diphosphate	0.0025	100–110
Phosphopyruvic acid	0.0025	70
3-Phosphoglyceric acid	0.0025	70
Fructose-1,6-diphosphate	0.0025	90
"	0.00125	65
Inosine triphosphate	0.0025	60
" diphosphate	0.0025	60
Phosphocreatine	0.025	30
Thiamine pyrophosphate	0.0025	10
Diphosphopyridine nucleotide	0.0025	5
Adenylic acid	0.0025	0
Adenosine	0.0025	0
Adenine	0.0025	0
Inosinic acid	0.0025	0
Hypoxanthine	0.0025	0
α- and β-phosphoglycerol	0.0025	0
" " " + choline	0.0025	0
" " " + " + ATP	0.0025	100
Fructose-6-phosphate	0.0025	0
Sodium acid phosphate	0.0025	0
" pyrophosphate	0.0025	0

The curves of oxygen uptake were not always linear and the activation due to the individual compounds varied somewhat with the age of the preparation, but the figures reported represent a fair average of the data obtained (see Table II).

The results are significant, since only certain phosphorylated compounds were active. Of the various degradation products of ATP (ADP, AMP (adenylic acid), adenosine, and adenine) only ADP was active. The activation by ADP seemed equivalent to that of ATP. When ATP and ADP were compared in concentrations giving equal amounts of acid-labile phosphorus, the activations were not identical, that of ADP being greater. Two facts concerning the activation by ADP should be pointed out in this connection. Unpublished data of Dr. G. A. LePage (personal communication) indicate that the adenosine polyphosphate found in quick frozen rat liver is predominantly ADP. Colowick and Kalckar (27) have stated that liver is devoid of myokinase (which catalyzes "phosphate dismutation" between 2 molecules of ADP, yielding 1 of ATP and 1 of AMP).

The inosine nucleotides (deamination products of corresponding adenosine nucleotides) behaved as expected, Kleinzeller (12) has shown that such nucleotides can substitute for adenosine nucleotides in phosphorylation reactions, but that the rate of the reactions is considerably lower. The lower metabolic products, inosinic acid and hypoxanthine, were inactive.

The activations by phosphopyruvic, 3-phosphoglyceric, and fructose-1, 6-diphosphate are of interest because these compounds are eventually at some stage in their metabolism capable of phosphorylating adenylic acid. Phosphopyruvic acid can perform this directly; the other two compounds are converted to phosphopyruvic acid in glycolysis. Since hexose diphosphate has two potential P groups, capable of phosphorylating adenylic acid, the data on two different concentrations of the compound fall into line with the suggestion made below.

Phosphocreatine is capable of phosphorylating adenylic acid. There is some question as to whether the compound exists in the liver (26). If it acts by phosphorylating adenylic acid, it seems somewhat less effective in the liver homogenate than it is known to be in muscle extracts. It was, however, quickly dephosphorylated in the liver preparation, as determined by analysis.

Thiamine pyrophosphate showed a small activation. This compound can apparently phosphorylate adenylic acid in yeast (28), but it is probably of no metabolic significance in these experiments, since its concentration in liver is relatively very low, compared to that of ATP.

The questionable results obtained with diphosphopyridine nucleotide may be attributed to impurities in the preparation (46 per cent pure).

Inorganic phosphate, pyrophosphate, phosphoglycerol, and fructose-6-phosphate did not activate the system. The latter two compounds are unable to phosphorylate adenylic acid directly.

Since ATP (or ADP) was the most active compound tested, the positive results with phosphopyruvic, phosphoglyceric, hexose diphosphate, and phosphocreatine can best be interpreted by assuming that these compounds acted through the formation of ATP by donating phosphorus to adenylic acid, which they are capable of doing during glycolysis. This interpretaion is confirmed in section (8).

Choline and phosphoglycerol, with or without ATP, showed absolutely no effect on the occurrence or rate of the oxidation. The lack of stimulation by these precursors of phospholipid suggested that activation by ATP did not necessarily lead to obligatory formation of phospholipid before oxidation.

Experiments recently reported by Leloir and Muñoz (29) indicate that phosphopyruvic acid may play a key rôle in the relationship between oxidation of fumarate and butyrate. Their observations on the peculiar requirement of bicarbonate in the activation of butyrate oxidation by phosphopyruvate were not noticed in the rat liver preparations used in this study. Two different preparations of phosphopyruvate were used with identical results. The system used in this study is, however, more complex than that of Leloir and Muñoz. In general their data coincide with the suggestions elaborated in this paper.

8. Activation by Phosphorylation of Adenylic Acid—If phosphopyruvic acid, 3-phosphoglyceric acid, fructose-1,6-diphosphate, and phosphocreatine activate the fatty acid oxidation by phosphorylation of adenylic acid, then the addition of adenylic acid, which is itself inactive, to a system containing the individual compounds above should enhance the activation by such compounds. In experiments to demonstrate this point the systems were made up exactly as in section (7), with and without the addition of adenylic acid in a final concentration of 0.00125 M. ATP was of course omitted.

The data, in Table III, show that the addition of adenylic acid does enhance the activation due to the compounds listed above. Adenylic acid is completely inactive by itself. The results provide confirmatory evidence for the tentative conclusions of section (7).

The activation is not quantitative, in the sense that potentially the system should show 100 per cent activation, because the compounds involved are capable of other reactions and are at all times susceptible to dephosphorylation by phosphatases.

This stimulation by adenylic acid was most striking in preparations aged 4 to 6 hours. Such treatment was found by experiment to result in deamination of adenylic acid to inosinic acid and also some dephosphorylation of both compounds. The activation by phosphopyruvic acid, etc., is greatly decreased by this treatment but it can be restored considerably by addition of adenylic acid.

9. Activation of Fatty Acid Oxidation by Aerobic Phosphorylation of

Adenylic Acid—If the conclusions above are true, then a system in which some carbohydrate metabolite is being actively oxidized should also activate fatty acid oxidation by phosphorylating adenylic acid to provide enough ATP for this function. Aerobic phosphorylation in such systems has been described by Colowick *et al.* (7), Ochoa (8), and others.

In the experiments in Table IV the data show a high rate of O_2 uptake in the presence of pyruvate and fumarate. This rate was increased by addition of octanoate, even though no ATP had been added. The omission of the pyruvate or fumarate resulted in the usual inhibition by octanoate. These experiments completely bear out the conclusions from the previous sections.

10. Oxidation of Normal Saturated Fatty Acids—In these experiments the oxygen uptake of the system as described in section (4), in the presence of the normal saturated fatty acids with and without ATP activation, was studied. The liver homogenates were aged 4 to 6 hours. Acids containing up to 8 carbon atoms were present in a concentration of 0.007 M; the higher acids were 0.002 M. In these experiments the extra oxygen uptake due to octanoate oxidation in the presence of ATP was used as a standard and assigned a value of 100. The extra uptake or inhibition of the other acids was expressed in terms of this standard (see Table V).

TABLE III

Effect of Adenylic Acid in Increasing Activation of Octanoate Oxidation by Some Phosphorylated Compounds

Activation by adenosine triphosphate (ATP) is given the value of 100. See section (8) for the details.

Activator	Activation	
	Fresh preparation	Aged preparation
ATP ..	100	100
Phosphopyruvic acid	70	25
" " + adenylic acid	85	70
Phosphoglyceric "	70	40
" " + adenylic acid	80	70
Phosphocreatine	30	10
" + adenylic acid .	45	35

The data show that all the acids require activation by ATP except possibly acetic and propionic acids, which are oxidized somewhat without the addition of ATP, and formic acid, which is not oxidized with or without ATP. It is indicated that these lower acids are perhaps not oxidized by the same mechanism as the higher acids. There is a progressive rise in uptake with increase in length of the chain, but since the products and mechanism of the oxidations are not known it is difficult to assess the relative necessity of ATP for the different acids. However, it is clear that the *inhibition* of the endogenous respiration *without ATP* becomes greater with increase in length of the chain to the stage of octanoic acid. In the sense of preventing such inhibition, however, ATP is more necessary as the chain length increases.

There was no great difference in rate of oxidation of the odd carbon acids from that of next homologues, although it is known that they give rise to different products (1).

The successful experiments with acids having more than 8 carbon atoms is of significance, since the oxidation of the biologically predominant 16- and 18-carbon acids can now be approached experimentally.

TABLE IV

Activation of Octanoate Oxidation by Aerobic Phosphorylation

The system contained 170 mg of homogenized liver tissue, final concentrations of 0.00125 M adenylic acid, 1×10^{-4} M diphosphopyridine nucleotide, 0 01 M $MgCl_2$, 0.02 M pyruvate (or 0.02 M fumarate) (or water), 4×10^{-5} M cytochrome, 0.03 M phosphate, 0.021 M NaCl, 0 01 M KCl, and 0.007 M octanoate (or water) in a total volume of 2.0 ml. Adenosine triphosphate was not added Time, 30 minutes.

System	Octanoate	O_2 uptake
		c mm
Complete (pyruvate)	−	160
" "	+	189
" without pyruvate	−	128
" " "	+	100
" (fumarate)	−	146
" "	+	186
" without fumarate	−	120
" " "	+	114

The inhibition of endogenous respiration by fatty acids in the absence of ATP is significant, because it tends to follow the known facts concerning the capillary activity and adsorption of the fatty acids and soaps (24). The free acids and soaps both depress surface tension, the free acids being more active in this regard up to 8 carbon atoms. With increase in chain length beyond 8 carbon atoms, the surface activity of the free acids diminishes because of reduced solubility, while that of the soaps increases somewhat. Since at pH 7.4 the higher acids exist as a mixture of acid and soap, the data become difficult to interpret but in general the inhibition can be explained on the basis of surface activity.

The importance of the proper colloidal condition of the substrate mixture of free acid and soap in the case of 16- and 18-carbon compounds for availability to the enzyme is shown by the three oxidation values assigned to stearic acid (Table V). The first, 35, was obtained with a solution of stearate neutralized in the cold; the other two figures were obtained when the solution was neutralized at 60° and allowed to cool. This consideration of the colloidal state of the lipid must be kept in mind, when such substrates are used, and again calls attention to the technical difficulty of providing the proper colloidal conditions for the enzymes involved.

11. Organic and Inorganic Phosphate Exchanges—Obviously, the analysis of the different phosphate fractions in the system described under different conditions would be a desirable experimental approach in confirming the ATP activation and in determining its mechanism. Actually the results obtained have not been particularly illuminating. The reasons for this can be predicted from data of previous sections. The endogenous respiration was found to be capable of performing aerobic phosphorylation (differences between aerobic and anaerobic experiments). The liver extracts contained very active phosphatases (0.001 M ATP was completely dephosphorylated in 9 minutes with cyanide inhibition of respiration). Difficulties of this sort were encountered by Ochoa (8) in determining the efficiency of aerobic phosphorylation. Also, in order to detect very small

TABLE V

Oxidation of Normal Saturated Fatty Acids with and without Activation by Adenosine Triphosphate (ATP)

See section (10) for the details The extra uptake in the presence of octanoic acid was the standard assay value and assigned a value of 100. Plus values indicate extra uptake, negative values extent of inhibition of endogenous respiration, 0 indicates no oxidation or inhibition. Time, 50 minutes

Acid	Oxidation without ATP	Oxidation with ATP
$HCOOH$	0	0
CH_3COOH	+30	+30
C_2H_5COOH	+10	+30
C_3H_7COOH	−5	+50
C_4H_9COOH	−30	+65
$C_5H_{11}COOH$	−55	+80
$C_6H_{13}COOH$	−55	+80
$C_7H_{15}COOH$	−60	+100
$C_8H_{17}COOH$	−65	+100
$C_9H_{19}COOH$	−60	+100
$C_{11}H_{23}COOH$	−50	+100
$C_{13}H_{27}COOH$	−50	+90
$C_{15}H_{31}COOH$	−40	+110
$C_{16}H_{33}COOH$	−40	+90
$C_{17}H_{35}COOH$	−35	+35, +90, +100

differences in the organic phosphate fractions unsuccessful attempts were made to substitute other buffers for the high concentrations of phosphate buffer used. The only useful buffer was borate which, however, inhibited extra oxygen uptake 40 per cent. The complex-forming power of borate is well known. In general, the interplay of these factors did not permit the discovery of any significant differences in organic phosphate fractions or in hydrolysis curves (1 N HCl at 100°). The changes noted by Muñoz and Leloir in their partially purified system could be confirmed in their system but were obscured in the rat liver homogenate. Obviously, some of the interfering factors have been removed in their system.

Some data worthy of note were obtained, however. It was found that analyses of labile P (7 minutes hydrolysis) in the experiments of sections (4) to (9) (at 10 minutes) in all cases showed concentrations (calculated as ATP) of at least 1×10^{-4} M in all experiments in which octanoate was being oxidized. Greatest concentrations (0.001 M and higher) were noted when ATP or phosphopyruvic acid + adenylic acid activated the systems, least in the aerobic phosphorylation experiments. Conversely, it was found that conditions which did not allow octanoate oxidation showed analyses of less than 1×10^{-4} M or no labile P. These data confirmed the finding that an initial concentration of at least 1×10^{-4} M ATP was necessary to detect extra oxygen uptake in the presence of octanoate.

If a phosphorylated fatty acid is the result of ATP activation, such a product will undoubtedly be quite labile and until something can be learned of the properties of this intermediate by other means detection in reaction media will probably prove to be difficult.

It is hoped that consideration of the working hypothesis elaborated in the following discussion will prove helpful in studying the mechanism of the activation by allowing certain crucial experiments to be performed, perhaps on partially purified systems. It is felt that the crude homogenates used in this study may have outlived their usefulness in an examination of the activation mechanism, because of the complexity of interfering reactions.

DISCUSSION

It is apparent from the experiments reported here that the least common denominator of all agencies leading to activation of fatty acid oxidation is ATP (or ADP). Since these compounds are most active of all those tested, and since the adenylic acid system is the point of mediation of most transphosphorylations, it is not unlikely that we are dealing with a phosphorylation of the fatty acid as an obligatory step in its oxidation.

The normal saturated fatty acid offers but one point of reactivity for such a process; namely, the carboxyl group. The suggestion offered by

Lipmann and Perlmann (30) that crotonic acid may be phosphorylated, then dehydrogenated, and dephosphorylated to yield enol-acetoacetate (which they proposed to account for the production of acetoacetate from crotonate without β-hydroxybutyrate as intermediate) is untenable in this situation. Such a mechanism for explaining activation by ATP presupposes a preliminary dehydrogenation to an unsaturated acid *before* the action of the phosphorylating agent. In these experiments no such dehydrogenation (as evidenced by extra oxygen uptake in the presence of fatty acid *without* ATP) was found. The activation by ATP is primary to the whole oxidation mechanism. This leaves the acyl phosphate of the fatty acid as the most likely product of a transphosphorylation.

There are two known cases of the formation of acyl phosphates as intermediates. Acetyl phosphate has been demonstrated to be an intermediate in pyruvate oxidation by *Bacterium delbruckii* by Lipmann (31). The other case is that of the R-diphosphoglyceric acid, formed in the course of triose phosphate oxidation, which has been shown by Negelein and Brömel (32) to be 1,3-diphosphoglyceric acid. These compounds are capable of phosphorylating either adenylic acid or ADP respectively. The reversibility of these reactions has been demonstrated only in the latter case (33, 6) but this positive demonstration furnishes at least some thermodynamic basis for the possibility of acyl phosphorylation suggested by the experiments of this paper. The formation of an acyl pyrophosphate is another possibility. Unpublished work by Cori, Ochoa, and Cori indicates a synthesis of inorganic pyrophosphate in liver extracts coupled with pyruvate oxidation (8). Ochoa suggests this may arise from the decomposition of an acyl pyrophosphate, which can be predicted to be unstable at the carbonphosphorus linkage.

The formation of an acyl phosphate as a first step in oxidation seems entirely parallel to the hexokinase reaction (phosphorylation of glucose by ATP). As a matter of fact the parallel has been drawn further by the suggestion of Lipmann (34) that fatty acid phosphates may well be the intermediates for the synthesis of the glyceride bond. By extension, these suggestions imply a parallel between the first stages of fat and carbohydrate metabolism, the former being analogous to the phosphorylase-hexokinase reactions for making glycogen or glucose available for the proper enzymatic channels as hexose phosphate.

There is further presumptive evidence for the formation of an acyl phosphate in these experiments. The inhibition of endogenous respiration by fatty acids without ATP and the inhibition of respiration caused by excess fatty acid even in the presence of ATP take on some significance, because ATP is apparently able to neutralize the non-specific surface activity of the fatty acids and prevent such inhibition. Such a change in surface activity of the fatty acid may well result from the formation of an acyl phosphate. It can be predicted, from certain data in the literature, that such a compound would be more highly dissociated, more soluble, more nearly hydrophilic, and show less surface activity than the fatty acid or soap itself. The data on the inhibition of respiration by the different fatty acids without ATP show that such inhibition is parallel to the known surface activity of these compounds and that in this sense ATP is more necessary for activation as the chain length increases. It is not to be concluded that ATP is necessarily required to prevent such surface activity of free fatty acids in the cell itself, but that this phenomenon is a peculiar feature of *in vitro* experimentation which may yield information concerning the mechanism.

A final point of evidence is the fact observed by Muñoz and Leloir (3) that the phosphate changes they observed took place in the presence of the higher fatty acids even though there was no oxidation of these acids. This observation also serves to divorce the primary phosphorus exchanges from the oxidation of the fatty acid, a corollary of the hypothesis stated here.

It might also appear that the obligatory formation of phospholipid is necessary before oxidation takes place. This seems unlikely, especially in the case of the shorter chain acids, but if it is true it is still conceivable that the acyl phosphate is the first step in such a synthesis, according to Lipmann's suggestion (34).

The high energy phosphate bond represented by the acyl phosphate is in no sense to be considered a net loss from the ATP reservoir, since it may well be returned to the latter at some later stage in the catabolism of the fatty acid, along with whatever high energy phosphate bonds are generated by the oxidations themselves.

In view of the evidence and arguments presented here and also considering the fact that such an interpretation does not conflict with the present state of knowledge concerning metabolic patterns involving phosphorus, it is believed that the most reasonable working hypothesis for the activation of fatty acid oxidation by ATP is the obligatory formation of the acyl phosphate bond.

Work in this laboratory is continuing along the lines of this hypothesis. Pure crystalline fatty acid phosphates having from 8 to 16 carbon atoms have been synthesized in this laboratory and these compounds and their rôle as intermediates form the subject of following communications.

The author wishes to acknowledge the encouragement of his colleagues, Professors H. C. Bradley, E. J. Witzemann, and P. P. Cohen, in the course of this investigation.

SUMMARY

1. The oxidation of fatty acids by broken cell preparations of rat liver requires the presence of adenosine polyphosphates. This fact explains the failure or only partial success of previous investigators to realize this process *in vitro*.

2. The rate of oxidation of the fatty acid in the system described depends directly on the concentration of adenosine triphosphate. The optimum concentration of adenosine triphosphate required is approximately that existing in the liver cell *in vivo*.

3. The activation of the oxidation can also be shown if the adenosine polyphosphates are generated *in situ* by phosphorylation of adenylic acid by suitable phosphate donors, or by aerobic phosphorylation during the course of pyruvate or fumarate oxidation.

4. All the normal saturated fatty acids having from 4 to 18 carbon atoms are oxidized in this system, as evidenced by the increase in oxygen uptake, but only in the presence of adenosine polyphosphates.

5. These observations, together with certain other data reported, have been correlated into a working hypothesis concerning the mechanism of the activation, a central feature of which is the possible formation of fatty acid acyl phosphates as intermediates.

BIBLIOGRAPHY

1 Quastel, J H , and Wheatley, A H M , *Biochem J.*, **27**, 1753 (1933).
2 Leloir, L F., and Muñoz, J M , *Biochem. J* , **33**, 734 (1939)
3 Muñoz, J M , and Leloir, L F , *J Biol. Chem* , **147**, 355 (1943)
4. DuBois, K P , and Potter, V R , *J Biol Chem* , **150**, 185 (1943)
5. Kalckar, H , *Enzymologia*, **2**, 47 (1937)
6 Warburg, O , and Christian, W , *Biochem Z* , **303**, 40 (1939)
7. Colowick, S. P , Kalckar, H. M , and Cori, C F , *J. Biol Chem* , **137**, 343 (1941).
8. Ochoa, S., *J. Biol Chem.*, **151**, 493 (1943).
9. DuBois, K P , Albaum, H G., and Potter, V R , *J Biol Chem* , **147**, 699 (1943).
10. Needham, D M , *Biochem J.*, **36**, 113 (1942)
11. Bailey, K , *Biochem. J.*, **36**, 121 (1942).
12. Kleinzeller, A , *Biochem J* , **36**, 729 (1942)
13 Zeile, K , and Fawaz, G , *Z physiol Chem* , **256**, 193 (1938).
14 Fiske, C H , and Subbarow, Y , *J Biol Chem* , **81**, 629 (1929)
15. Kiessling, W , *Ber chem Ges* , **69 B**, 2331 (1936).
16. Williamson, S , and Green, D. E., *J. Biol. Chem* , **135**, 345 (1940).
17 Keilin, D , and Hartree, E. F , *Proc. Roy. Soc. London, Series B*, **122**, 298 (1937).
18. Ostern, P , *Biochem Z* , **254**, 65 (1932).
19. Fiske, C H , and Subbarow, Y , *J. Biol. Chem.*, **66**, 375 (1925).
20. Potter, V. R., and Elvehjem, C. A., *J. Biol. Chem.*, **114**, 495 (1936).
21. Krebs, H. A., *Z. physiol. Chem.*, **217**, 191 (1933).
22. Bernheim, F., *J. Biol Chem* , **133**, 291 (1940).
23. Velluz, L , *Bull Soc chim. biol* , **9**, 483 (1927)
24. Jarisch, A , *Biochem. Z.*, **134**, 163 (1922).

25. Toropova, G., *Biokhimiya*, **7**, 32 (1942).
26. Flock, E., Bollman, J. L., and Mann, F. C., *J. Biol. Chem.*, **115**, 179 (1936).
27. Colowick, S. P., and Kalckar, H. M., *J. Biol. Chem* , **148**, 117 (1943).
28. Lipschitz, M., Potter, V. R , and Elvehjem, C. A , *Biochem J.*, **32**, 474 (1938).
29. Leloir, L. F., and Muñoz, J. M , *J. Biol. Chem.*, **153**, 53 (1944).
30. Lipmann, F., and Perlmann, G. E , *Arch. Biochem.*, **1**, 41 (1942).
31. Lipmann, F., *Federation Proc* , **1**, pt. 2, 122 (1942).
32. Negelein, E., and Brömel, H., *Biochem. Z.*, **301**, 135 (1939); **303**, 132 (1939).
33. Meyerhof, O., Ohlmeyer, P , and Mohle, W., *Biochem Z.*, **297**, 113 (1938).
34. Lipmann, F., in Nord, F F , and Werkman, C H., Advances in enzymology and related subjects, New York, **1**, 99 (1941).

THE USE OF NEUROSPORA FOR THE DETERMINATION OF CHOLINE AND BIOTIN IN MILK PRODUCTS

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A simplification of the *Neurospora* method (1) for choline has been found very useful in our laboratory when applied to dairy products. Erlenmeyer flasks of 50 ml. capacity are used for the mold growth. In these are placed 5 ml. of double strength media, the desired amount of the choline-containing extract or solution, and sufficient distilled water to make a total of 10 ml. A growth period of 5 days is used. The permutit step has been eliminated, since it was quite time-consuming. This introduced the question of whether methionine (1), hydrolyzed casein, or lactose would stimulate or inhibit the growth of the *cholineless Neurospora crassa*, since these substances would be eliminated by the permutit step. A brief study of this question has been made.

In the study the ratio of choline to the suspected interfering substance has been kept constant, since this is the manner in which they would occur in a given sample of the unknown. The ratios used were choline to methionine 1:4, choline to methionine 1:50, choline to lactose to hydrolyzed casein 1:200:200, choline to hydrolyzed casein 1:200, and choline to lactose 1:200. With the exception of the higher choline to methionine ratio these are approximations of those in which choline and the suspected interfering material occur in milk. Growth on a double strength media was also investigated.

The results of the study are presented in Table I. Each value represents the average of two duplicate determinations in one trial. Data for one or more and usually for two or three trials are given. At the 1:50 ratio methionine interfered even at low assay levels. None of the other treatments produced an interference over the steep portion of the assay curve; *i.e.*, below a choline value of 5.0 γ. At the break in the assay curve or at the plateau results are inconsistent and unreliable in the presence of methionine, of hydrolyzed casein and lactose, or of hydrolyzed casein alone. It is therefore imperative that assay values be derived from the steep

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portion of the standard curve when the simplified method is used. Lactose by itself is relatively inert for the organism, as is shown in Table I. When it is used to replace sucrose in the medium, only a slight growth occurs in the presence of adequate biotin and choline.

TABLE I

Per Cent Choline Recovered in Presence of Suspected Interfering Materials

Choline level per flask	Ratio of choline to methionine, 1 4	Ratio of choline to methionine, 1 50	Ratio of choline to lactose to hydrolyzed casein, 1 200.200	Ratio of choline to hydrolyzed casein, 1.200	Ratio of choline to lactose, 1 200	Choline in double strength media
γ						
1.0	95, 88	125, 110	80, 80	95, 105		80
2 5	88, 100, 94	116, 204, 198	96, 100, 92	80, 92, 86	100	80, 120, 100
3.5	86, 96	175, 236	86, 80	92, 111		119, 106
5.0	112, 101, 100	130, 167, ∞*	94, 81, 81	84, 85, 106	104	106, 142, 144
6.0	111, 120	272, ∞	62	85, 99		170, 125
7.5	100, 98, 103	111, 346, ∞	90, 80, 72	80, 81, 104	96	125, 367, ∞
10.0	80, 107, 126	110, 400, ∞	68, 95, 61	70, 94, 98	98	135, 400, ∞
15.0	58, 125, 250	180, ∞, ∞	59, 54, 141	55, 213, ∞	82	190, ∞, ∞

* Data beyond range of standard assay curve.

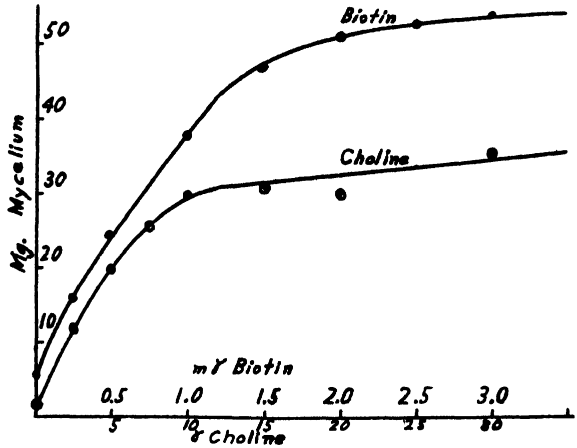

FIG. 1 Growth response of *cholineless* to choline and to biotin

If the concentrations of the ingredients of the media are doubled, erratic response to choline often results. The reason for this is not clear. However, it is not believed to be a source of error in the assay. Because of the sensitivity of the organism to choline and biotin the amount of salts and soluble solids from milk added to the test media in the assay is rather small in comparison to those already present.

The data in Table I indicate that satisfactory results within ±20 per cent are obtained with the simplified method. Recoveries of choline added to eight samples of milk varied from 85 to 102 and averaged 96 per cent. While the range of recoveries is not quite as good as those found by Horowitz and Beadle (1) or by Luecke and Pearson (2) with *Neurospora* methods for choline, the average result is quite comparable. When ten samples of fresh milk were assayed by the simplified method, the average result, 149 mg. per liter, was in excellent agreement with the average data, 147 mg. per kilo, secured by Engle (3) with a chemical method.

Biotin—The *cholineless Neurospora crassa* has also been used for biotin assays on milk and other dairy products. The wild type of *Neurospora* might be more suitable for this purpose, since biotin would be its only vitamin requirement, but the use of the wild type would require the maintenance of a separate culture.

When *cholineless* is used for biotin assays, the biotin in the media is replaced by 20 mg. of choline per liter of the double strength media. The sample preparation is the same as used for the choline assay (1) with the omission of the permutit step. This permits one to use the same extract for both assays. The response of the organism to graded quantities of biotin is good and the assay curve is steep over a wider range of biotin increments than is a similar curve for choline (Fig. 1). Assay values for milk, obtained from various levels of the curve, agree very well. Assays on ten samples of fresh milk indicate that most of the values fall in the range of 30 to 40 millimicrograms per ml., which are similar to the results found by Lampen, Bahler, and Peterson (4) with *Clostridium butylicum*. The wild type of *Neurospora crassa* and presumably *cholineless* responds to desthiobiotin as well as biotin (5) but this compound is not known to be present in milk. The response of *Neurospora* to other compounds related to biotin has not as yet been investigated as it has for *Lactobacillus casei* and *Saccharomyces cerevisiae* (6).

SUMMARY

1. The permutit step in the *Neurospora* assay for choline in milk products may be omitted if the assay data are taken only from the lower steep portion of the assay curve.

2. The same organism and the same extracts may be used for biotin assays.

BIBLIOGRAPHY

1. Horowitz, N. H., and Beadle, G. W., *J. Biol. Chem.*, **150**, 325 (1943).
2. Luecke, R. W., and Pearson, P. B., *J. Biol. Chem.*, **153**, 259 (1944).
3. Engle, R. W., *J. Nutr.*, **25**, 441 (1943).
4. Lampen, J. O., Bahler, G. P., and Peterson, W. H., *J. Nutr.*, **23**, 11 (1942).
5. Lilly, V. G., and Leonian, L. H., *Science*, **99**, 205 (1944).
6. Dittmer, K., and du Vigneaud, V., *Science*, **100**, 129 (1944).

INVESTIGATIONS OF AMINO ACIDS, PEPTIDES, AND PROTEINS

XX. THE DETERMINATION OF APPARENT FREE TRYPTOPHANE IN BLOOD BY A MICROBIOLOGICAL METHOD*

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Tryptophane is indispensable for the growth and the maintenance of optimal metabolic functions of animals; yet it has not been possible to determine completely the physiological rôle of this amino acid. That present knowledge is incomplete may be explained in part by lack of an analytical tool with which the tryptophane content of body organs and fluids may be measured conveniently and accurately. Previous studies have been limited largely to the determination of tryptophane by colorimetric methods.

A microbiological procedure which is considered to be satisfactory for the determination of apparent free tryptophane in blood is described in the present paper. Analogous procedures for the determination of tryptophane in mixtures of pure amino acids have been described by Shankman, Dunn, and Rubin (2), and a method for the determination of tryptophane in protein hydrolysates has been reported by Greene and Black (3). The present authors' standard curves for $l(-)$-tryptophane were similar in all respects to those obtained previously by Shankman (4) with media containing either a mixture of pure amino acids or an acid hydrolysate of casein supplemented with cystine.[1] A nearly identical standard curve was obtained by Greene and Black (3). The observation of these investigators that $d(+)$-tryptophane is not utilized by *Lactobacillus arabinosus* has been confirmed by the present authors (unpublished data). Enzyme and alkali hydrolysates of casein were assayed by Greene and Black who found 1.15 per cent of tryptophane in casein containing 15.6 per cent nitrogen. This value is about 25 per cent below the figure, 1.54

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[1] Private communication from Dr. S. Shankman.

per cent (5), which has been commonly, although probably incorrectly, regarded as correct.

EXPERIMENTAL[2]

Blood samples (about 50 ml.) were collected at 10.00 a.m. from an arm vein of eleven normal males who had fasted about 15 hours. When plasma was desired the receiving flask was coated with about 60 mg. of potassium oxalate anticoagulant. Blood serum and plasma were prepared by centrifuging coagulated or oxalated blood within an hour after the sample was drawn. The plasma or serum was deproteinized by adding with constant agitation 16 ml. of water, 2 ml. of 0.6 N sulfuric acid, and 2 ml. of 10 per cent sodium tungstate solution to 4 ml. of the liquid. The filtrate was brought to pH 7.0 by the addition of 1 N sodium hydroxide, with a Beckman pH meter to determine the end-point.

Amino nitrogen was determined by the method of Hamilton and Van Slyke (7) and tryptophane was determined by the authors' microbiological procedure with the assay technique described in previous publications. The composition of the basal medium is given in Table I. A series of twenty-five duplicate and triplicate standard curves was prepared, all of which resembled closely the standard curves obtained by Shankman (4) and Greene and Black (3). The experimental data and values calculated from the assays are given in Tables II to V.

DISCUSSION

The presence of free tryptophane in deproteinized blood was first reported in 1913 by Abderhalden (10) who detected traces of this amino acid in the tryptophane-mercuric sulfate precipitate by means of the glyoxylic acid and bromine colorimetric tests. The concentration of apparent free tryptophane in cow blood and plasma, deproteinized by coagulation with acetic acid, was determined in a series of careful experiments by Cary and Meigs (11, 12), reported in 1924 and 1928. Tryptophane was determined by colorimetric analysis of the product formed by the reaction of the tryptophane-mercuric sulfate complex with the *p*-dimethylaminobenzaldehyde or the Hopkins-Cole glyoxylic acid-sulfuric acid reagent. In their first experiments it was found that the apparent free tryptophane of cow blood varied from about 0.6 mg. per cent to 1.5 mg. per cent under different dietary conditions, even though there was little or no change in the level of total amino nitrogen as determined by the Van Slyke nitrous acid procedure. The apparent free tryptophane content of cow plasma obtained by their most reliable procedure varied from about 0.7 mg. per cent to about 1.3 mg. per cent, with an average value of 1.12 mg. per cent.

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[2] In preliminary experiments on the determination of tryptophane attempts were made to employ dialysates of plasma prepared with the apparatus described by Hamilton and Archibald (6) Because the values for tryptophane in the dialyzed plasma and the rate of dialysis of pure *l*(−)-tryptophane were very low, the dialysis technique was temporarily abandoned. Tungstic acid was selected as the protein precipitant because of the observation by Hamilton and Van Slyke (7) that it gave a quantitative yield of α-amino nitrogen according to ninhydrin analysis. Furthermore, tungstic acid yields a nearly neutral filtrate since almost all of the acid is adsorbed by the protein precipitate (8).

TABLE I

*Composition of Basal Medium**

Constituent	Amount per 100 ml
l(−)-Cystine,† *mg*	12
Casein hydrolysate,‡ *ml*	6.66
Glucose,§ *gm*	2.0
Sodium acetate,§ *gm*	1.2
Adenine sulfate,‖ *mg*	1.2
Guanine hydrochloride,‖ *mg*	1.2
Uracil,‖ *mg*	1.2
KH_2PO_4,§ *mg*	54
K_2HPO_4,§ *mg*	54
$MgSO_4\cdot 7H_2O$,§ *mg*	21.6
$FeSO_4\cdot 7H_2O$,§ *mg*	1.08
$MnSO_4\cdot 4H_2O$,§ *mg*	1.08
Thiamine,¶ γ	40
Pyridoxine,¶ γ	64
dl-Calcium pantothenate,¶ γ	80
Riboflavin,¶ γ	80
Nicotinic acid,¶ γ	80
Biotin,** γ	0.2
p-Aminobenzoic acid,¶ γ	4

* The composition of the basal medium was similar to but not identical with that of Snell and Wright (9). Stock solutions of the vitamins in 50 per cent ethanol and other constituents in water or 1 N HCl were employed.

† Amino Acid Manufactures' product.

‡ Prepared according to the method of Snell and Wright (9).

§ C.P. grade.

‖ Eastman Kodak Company's product.

¶ Gelatin Products Company's product.

** Merck and Company's crystalline product.

Confidence in the reliability of these data was strengthened by the investigations of Cary (12), who determined by spectrophotometric and other means the optimal conditions for the deproteinization of plasma, the precipitation of the tryptophane-mercuric sulfate complex, and the colorimetric analysis for tryptophane, and who reported recoveries of tryptophane ranging from 98.6 to 101.4 per cent from known solutions containing 0.7 mg. per cent of pure tryptophane. On the other hand it was estimated that "the actual tryptophane originally present in blood is . . . 15 to 25 per cent less than the colorimetric results would indicate," since plasma filtrates were shown to contain a "foreign" yellow pigment which would be determined as tryptophane in the colorimetric analysis.

TABLE II

Results of Assay of Tryptophane in Tungstic Acid Filtrate of Blood Plasma

Diluted tungstic acid filtrate of plasma per tube*	Titration volume of 0.0213 N NaOH per tube	Tryptophane† found	
		Per tube	Per ml sample
ml	*ml*	γ	γ
0.20	2.89	0.39	1.95
0.20	2.85	0.38	1.90
0.40	4.57	0.73	1.82
0.40	4.78	0.77	1.92
0.60	5.60	1.05	1.75
0.60	5.65	1.08	1.80
0.80	6.44	1.50	1.87
0.80	6.52	1.55	1.93
1.00	7.08	1.88	1.88
1.00	7.14	1.92	1.92

* The solution for each assay was prepared by adding 1.00 ml. of the basal medium, 0.20 to 1.00 ml. of the tungstic acid filtrate of plasma, and sufficient distilled water to make the final volume of the solution 2.00 ml. The resulting acid was titrated by using standard approximately 0.020 N sodium hydroxide and bromothymol blue indicator or the Beckman pH meter.

† An average of 1.87 γ of tryptophane was found per ml. of the diluted tungstic acid filtrate of plasma. The mean deviation of the values at the different levels was 2.7 per cent and the tryptophane content of the undiluted plasma was 1.10 mg. per cent. The tryptophane content of plasma obtained from ten males 23 to 39 years of age ranged from 0.85 to 1.30 (average 1.14) mg. per cent. The mean deviation of the values at the different levels ranged from 1.8 to 7.0 (average 3.6) per cent. Variations in tryptophane ranging from 0 to 6 (average 3) per cent were obtained with different tungstic acid filtrates of the same plasma and, in one case, with a sample of plasma which had stood for 1 week in the refrigerator.

It is of particular interest that the apparent free tryptophane of plasma (1.14 mg. per cent) and of serum (1.21 mg. per cent) observed in this laboratory was less than 1 per cent of the total tryptophane (181 mg. per cent) of serum (13), and that the ratio of the values for free tryptophane was about the same as that for α-amino nitrogen in plasma (4.18 mg. per cent) and serum (4.77 mg. per cent). Comparable values for the α-amino nitrogen of plasma and serum were reported by MacFadyen (14) and by

Hamilton and Van Slyke (7), who concluded that amino acids are set free by unknown reactions which occur when blood coagulates.

The apparent free tryptophane of cells (0.24 mg. per cent) was only about one-fifth that of plasma or serum, while the total α-amino nitrogen of deproteinized solutions prepared from laked cells (7.70 mg. per cent) was more than 1.5 times that of plasma or serum. It appears to be true, nevertheless, that the ratio of apparent free tryptophane in cells to the α-amino nitrogen in cells resulting from free amino acids is about the same

TABLE III

Recovery of l(−)-Tryptophane from Tungstic Acid Filtrate of Blood Plasma

Recovery solution*	Titration volume of 0.0213 N NaOH per tube	Tryptophane† found	
		Per tube	Per ml sample
ml	*ml*	γ	γ
0.20	1.30	0.27	1.35
0.20	1.31	0.27	1.35
0.40	2.41	0.56	1.40
0.40	2.39	0.55	1.37
0.60	3.31	0.81	1.35
0.60	3.38	0.84	1.40
0.60	3.40	0.84	1.40
0.80	3.90	1.05	1.31
0.80	4.00	1.09	1.38
1.00	4.43	1.35	1.35

* The diluted recovery solution was prepared by mixing 1.00 ml. of diluted tungstic acid filtrate from plasma and 1.00 ml. of a standard solution containing 1 γ of pure l(−)-tryptophane per ml.

† An average of 1.36 γ of tryptophane was found per ml. of diluted recovery mixture. The mean deviation of the values at the different levels was 1.6 per cent and the tryptophane recovery was 114 per cent. Recoveries of tryptophane in comparable experiments were 104 and 109 per cent, with 3.5 and 5.1 per cent deviations from the mean at the different levels. The recovery and deviation from the mean were 87 and 3.9 per cent, respectively, with a mixture prepared by adding 40 γ of l(−)-tryptophane to 4 ml. of plasma, deproteinizing as described, and diluting the tungstic acid filtrate to twice its volume

as the ratio of tryptophane to α-amino nitrogen in plasma or serum. This conclusion is based on the experiments of Danielson (15) who found that unlaked corpuscles of normal young men contain 1.04 (0.34 to 2.19) mg. per cent of amino nitrogen and that the higher amino nitrogen content of laked corpuscles is accounted for in large part by the liberation of nondiffusible glutathione. This apparent consistency in the ratio of free tryptophane to amino acid-amino nitrogen in plasma, serum, and cells supports the view of Danielson that "the amino acids present in the blood stream as a foodstuff are freely diffusible into and out of the red blood cells to the extent characteristic of the species." Fontes and Thivolle (16) have postulated that tryptophane plays a special rôle in hematopoiesis as the precursor of the tetrapyrrole group in hematin while Madden and Whipple (17) have shown that almost all of the amino acids are concerned in varying degrees with the formation of blood proteins. The present experiments give no indication that free tryptophane gives rise to the formation of blood protein in a manner disproportionate to its concentration in the plasma.

TABLE IV

Results of Assay of Tryptophane in Tungstic Acid Filtrate of Blood Serum

Diluted tungstic acid filtrate of serum per tube*	Titration volume of 0 0213 N NaOH per tube	Tryptophane† found	
		Per tube	Per ml sample
ml	*ml*	γ	γ
0.20	1.76	0.39	1.95
0 20	1.75	0 39	1.95
0.40	3.24	0.80	2.00
0.40	3.25	0.80	2.00
0.60	4.17	1.17	1.95
0 60	4.23	1.20	2 00
0.80	4 65	1.56	1.95

* The technique described in a foot-note to Table II for the assay of tryptophane in blood plasma was employed.

† An average of 1.96 γ of tryptophane was found per ml. of the diluted tungstic acid filtrate of serum The mean deviation of the values at the different levels was 1.10 per cent and the tryptophane content of the undiluted serum was 1.18 mg. per cent. The tryptophane found in the serum of five males ranged from 0.95 to 1.40 (average 1.21) mg. per cent. The mean deviations at the different levels ranged from 1 0 to 4.6 (average 3.0) per cent. Variations in tryptophane ranging from 0 to 8 (average 3) per cent were obtained with different tungstic acid filtrates of the same plasma. Recoveries of tryptophane were 84, 95, and 108 per cent The recovery was 114 per cent in one experiment in which tryptophane was added to plasma prior to the preparation of the tungstic acid filtrate

The determination of the degree of dependability of the authors' microbiological procedure and the reliability of the tryptophane data are considerations of prime importance in the present work. The assay technique and data are considered to be of relatively high precision, since there were small mean deviations from the mean of the values for tryptophane found per ml. of sample at the different levels of blood filtrates and there was no detectable drift in the titrations.

It is more difficult to assess the probable accuracy of the assay data. While the average recoveries of tryptophane were 104 ± 13 per cent from plasma and 100 ± 14 per cent from serum, the accuracy of the assays could be either higher or lower than these values for reasons discussed in an earlier paper (1). It is undoubtedly fortuitous that the 1.14 mg. per cent of apparent free tryptophane found in human plasma in the present experiments is in such close agreement with the 1.12 mg. per cent found in cow plasma by Cary and Meigs. On the other hand, this concordance supports the view that both values are correct in order of magnitude, since it seems unlikely that "foreign" substances would exhibit identical, or even comparable, tryptophane activity by colorimetric and microbiological procedures.

TABLE V

*α-Amino Nitrogen and Tryptophane in Tungstic Acid Filtrates of Blood Plasma, Blood Serum, and Blood Cells**

Subject No. (male)	Age	Blood fraction	Total α-amino nitrogen†	Tryptophane		
					α-Amino nitrogen‡	
	yrs.		*mg. per cent*	*mg. per cent*	*mg. per cent*	*per cent of total α-amino nitrogen*
1	39	Plasma	4.56	1.07	0.0734	1.6
2	32	"	4.63	0.94	0.0642	1.4
3	26	"	3.93	1.02	0.0697	1.8
4	23	"	3.74	1.26	0.0862	2.3
5	24	"	4.14	1.18	0.0810	2.0
6	29	"	3.78	1.25	0.0859	2.2
7	23	"	4.35	1.06	0.0728	1.7
7	23	"	4.30	1.04	0.0714	1.6
1	39	Serum	4.69	1.14	0.0784	1.7
7	23	"	4.76	1.23	0.0845	1.8
7	23	"	4.87	1.22	0.0836	1.7
2	32	Cells, unwashed	7.45	0.30	0.0203	0.27
7	23	" washed	7.95	0.17	0.0116	0.15

* The blood cells were washed by suspending them in an equal volume of 1 per cent NaCl solution, centrifuging the suspension, and repeating this process once. Solutions of corpuscle material were prepared for analysis by adding 12 ml. of distilled water, 6.5 ml. of 0.6 N sulfuric acid, and 6.5 ml. of 10 per cent sodium tungstate solution to 5.0 ml. of washed or unwashed corpuscles.

† The average total α-amino nitrogen values were 4.18 (plasma), 4.77 (serum), and 7.70 (cells).

‡ The average value for the tryptophane α-amino nitrogen of the total α-amino nitrogen was about 1.8 per cent for both plasma and serum.

It is recognized, also, that anthranilic acid (3) has 30 to 50 per cent and indole (3) 60 to 90 per cent of the activity of tryptophane when determined by *Lactobacillus arabinosus*, and that the presence in blood of these and any other substances possessing tryptophane activity would vitiate the accuracy of a tryptophane assay in proportion to their activity and concentration. It does not seem probable, however, that these effects are pronounced, since it seems almost certain that the stimulation or inhibition of growth and of acid-producing capacity of the microörganism would be readily noted if there were an appreciable concentration of any synergistic or antagonistic substance.

SUMMARY

1. A microbiological procedure for the assay of apparent free tryptophane in the tungstic acid filtrates of blood fractions, which is believed to be satisfactorily precise, has been described.
2. It has been found that the apparent free tryptophane content of serum is about 10 per cent higher than that of plasma, that the apparent free tryptophane content of plasma or serum is less than 1 per cent of the total tryptophane of serum, and that the free tryptophane of cells is about one-fifth that of plasma or serum.
3. It has been shown that the α-amino nitrogen of serum is about 14 per cent higher than that of plasma and that the α-amino nitrogen of laked cells is more than 50 per cent higher than that of plasma or serum.
4. It has been concluded tentatively that the tryptophane values for plasma and serum are correct in order of magnitude and may be accurate to within about 10 per cent.
5. The present experiments have given no indication that free tryptophane functions disproportionately to its concentration in the formation of blood proteins.

BIBLIOGRAPHY

1. Dunn, M. S., Camien, M. N., Shankman, S., Frankl, W., and Rockland, L. B., *J. Biol. Chem*, **156**, 715 (1944).
2. Shankman, S., Dunn, M. S., and Rubin, L. B., *J. Biol. Chem*, **150**, 477 (1943); **151**, 511 (1943).
3. Greene, R. D., and Black, A., *Proc. Soc Exp. Biol and Med.*, **54**, 322 (1943).
4. Shankman, S, *J. Biol. Chem.*, **150**, 305 (1943).
5. Folin, O., and Looney, J. M., *J. Biol. Chem.*, **51**, 421 (1922).
6. Hamilton, P. B., and Archibald, R. M., *Ind. and Eng. Chem., Anal. Ed.*, **16**, 136 (1944).
7. Hamilton, P. B., and Van Slyke, D. D., *J. Biol. Chem.*, **150**, 231 (1943).
8. Peters, J. P., and Van Slyke, D. D., Quantitative clinical chemistry, Methods, Baltimore, 65 (1932).
9. Snell, E. E., and Wright, L. D., *J. Biol. Chem.*, **139**, 675 (1941).
10. Abderhalden, E., *Z. physiol. Chem.*, **88**, 478 (1913).
11. Cary, C. A., and Meigs, E. B., *J. Agr. Res.*, **29**, 603 (1924); *J. Biol. Chem.*, **78**, 399 (1928).
12. Cary, C. A., *J. Biol. Chem.*, **78**, 377 (1928).
13. Milone, H. S., and Sullivan, M. X., *Med. Annal. District of Columbia*, **7**, 335 (1938).
14. MacFadyen, D. A., *J. Biol. Chem.*, **145**, 387 (1942).
15. Danielson, I. S., *J. Biol. Chem.*, **101**, 505 (1933).
16. Fontes, G., and Thivolle, L., *Sang*, **4**, 658 (1930).
17. Madden, C. S., and Whipple, G. H., *Physiol. Rev.*, **20**, 194 (1940).

THE MICROBIOLOGICAL DETERMINATION OF AMINO ACIDS

II. GLUTAMIC ACID*

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After the development of methods for the determination of valine, leucine, and isoleucine (1) with *Lactobacillus arabinosus* 17-5 as the test organism, it was assumed that other amino acids essential for the growth of this organism could be determined by about the same procedure. However, it was soon discovered that glutamic acid is unique in its relationship to the growth of *Lactobacillus arabinosus*. Once this relationship was understood, the microbiological determination of glutamic acid became one of the most satisfactory assay methods which the authors have attempted so far.

Glutamic acid has been reported to be an essential nutrient for *Lactobacillus arabinosus* 17-5 by a number of investigators (1–6). According to Pollack and Lindner (3) glutamine and glutamic acid have equal growth-promoting activity for this organism. Shankman *et al.* (6) have used *Lactobacillus arabinosus* to determine glutamic acid. In our hands unsatisfactory results were obtained by an analogous procedure. The consistently satisfactory standard curves which had been obtained with valine, leucine, and isoleucine were not obtained with glutamic acid. The frequent failure of the organism to respond at all at the lower test levels of glutamic acid was particularly troublesome. The data suggested that glutamic acid is converted to some other metabolite before being utilized, and that this conversion is readily performed by the organism when the concentration of glutamic acid is relatively high or when vigorous growth has once been initiated. Evidence is presented which indicates that glutamine may be the active metabolite. The analytical method reported here employs a medium supplemented with glutamine.

EXPERIMENTAL

Materials and Methods

The assay medium is prepared by omitting glutamic acid from the complete medium previously described (1) and adding the equivalent of 0.25 mg. of glutamine per tube. Titration of the lactic acid formed after a

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* Presented before the Division of Biological Chemistry at the 108th meeting of the American Chemical Society at New York.

72 hour incubation period at 35° is used as a measure of the growth of the bacteria. Pure $l(+)$-glutamic acid is used for the preparation of the standard curves. The details of the procedures for culturing the organism and carrying out the assays have been previously reported (1). For accurate results it is essential that the contents of the tubes be thoroughly mixed by shaking before autoclaving. All of the standards and unknowns for any given test should be autoclaved at the same time. It has been found advisable to bring the tubes to room temperature immediately after autoclaving by placing the racks in cold water.

Preparation of Casein—Pure casein was prepared by repeated precipitation at the isoelectric point by the method of Van Slyke and Baker (7) as modified by Cohn and Hendry (8, 9).

Preparation of Egg Albumin—The first preparation of egg albumin was made from a commercial product by precipitating it twice with sodium sulfate and then coagulating the material in hot water. The second preparation was made from fresh eggs. A crystalline product was obtained by the method of Kekwick and Cannan (10). The material was then coagulated in hot water as described by Chibnall, Rees, and Williams (11).

Preparation of Peptonized Casein—100 mg. of casein were heated under a reflux condenser for 4 hours with 10 ml. of normal hydrochloric acid. The hydrolysate was neutralized with sodium hydroxide and diluted to a suitable volume.

Preparation of Hydrolysates for Analysis—100 mg. samples of purified proteins were hydrolyzed by heating under a reflux condenser for 24 hours with 10 ml. of 6 N hydrochloric acid. The hydrolysates were diluted to about 50 ml., filtered, and then concentrated to a syrup *in vacuo*. The residue was dissolved in water, adjusted to neutrality with sodium hydroxide (phenol red, internal indicator), and then diluted to a suitable volume.

Natural foodstuffs were first dried *in vacuo* for 5 hours at 95°, and then extracted with dry ethyl ether for 16 hours. The fat-free samples were hydrolyzed in the same manner as the purified proteins except that somewhat larger samples were generally used. Pyrrolidonecarboxylic acid and glutathione were likewise hydrolyzed with 6 N hydrochloric acid. The pyrrolidonecarboxylic acid was refluxed for 4 hours and the glutathione for 24 hours.

Effect of Adding Glutamine to Assay Medium

A typical glutamic acid curve obtained with the usual medium without glutamine is shown in Fig. 1, Curve 2. Very little growth occurred at glutamic acid levels below about 0.08 mg. per tube, while marked growth occurred at slightly higher levels. In different experiments the sharp break in the curve occurred at somewhat different glutamic acid concentrations. Agreement of duplicate tests in the lower region of the curves was frequently poor.

With the hypothesis in mind that glutamine might be the active metabolite, glutamic acid curves were prepared with media containing various amounts of glutamine. It was realized that most of the glutamine would be converted to pyrrolidonecarboxylic acid by autoclaving previous to inoculating the tubes. A concentration of glutamine was sought which would leave just enough after sterilizing to initiate growth in all tubes. The curves obtained in this manner were regular over their entire range.

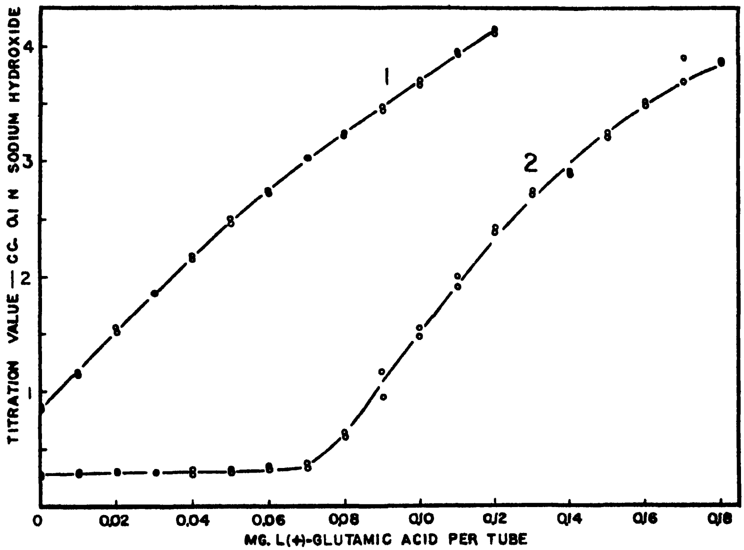

FIG 1. The effect of adding glutamine to the medium used for the assay of glutamic acid. Curve 1, 0.25 mg. of glutamine per tube; Curve 2, medium without glutamine. The titration values shown are for 5 ml. aliquots from 10 ml. cultures. The pH of the tubes when inoculated was 6.8.

Curve 1 of Fig. 1 shows a typical standard curve obtained with a medium containing the equivalent of 0.25 mg. of glutamine per tube. This level of glutamine has given consistently good results when the tubes were autoclaved at 15 pounds pressure for 15 minutes. Higher concentrations of glutamine gave high blanks. Tests were carried out in which the glutamine was sterilized by filtration and added aseptically to the tubes. In these tests smooth and regular glutamic acid curves of the type shown in Fig. 1, Curve 1, were obtained by adding only 0.02 mg. of glutamine per tube.

Pyrrolidonecarboxylic acid, asparagine, and ammonium salts were tested to determine whether any of these compounds could replace glutamine in the medium. The first two were entirely inactive. In the absence of glutamine, ammonium salts did increase the titration values. The extent of increase was dependent on the amount of glutamic acid in the tests and amounted to well over 50 per cent in some cases. The curves were not as satisfactory in these tests as those obtained by the use of glutamine. When glutamine was included in the medium, the further addition of as much as 5 mg. of ammonium chloride per tube did not cause a measurable change in the titration values. These findings can be readily explained on the basis that ammonium salts facilitate the formation of glutamine from glutamic acid by the organism.

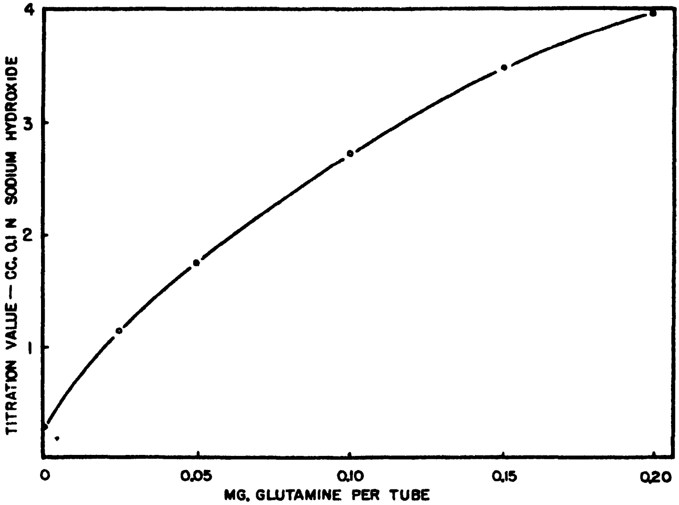

FIG. 2 The effect of glutamine on the growth of *Lactobacillus arabinosus* on a glutamic acid-free medium. In these tests the glutamine was not heated but sterilized by filtering through a Pyrex fritted glass filter of porosity UF. The titration values shown are for 5 ml. aliquots from 10 ml. cultures.

Fig. 2 shows the regular response of *Lactobacillus arabinosus* to graded amounts of glutamine on the glutamic acid-free medium. This behavior is in marked contrast to the response obtained with glutamic acid alone and suggests that glutamine is the active metabolite.

Specificity Tests

The authors' preliminary tests indicated that *dl*-glutamic acid was only half as active as *l*(+)-glutamic acid. However, Hac[1] found that the nonnatural form does have some activity. When rechecked, this proved to be the case (Table I). The ratio of the activity of *dl*- to *l*(+)-glutamic acid depends on the test level. For this reason it is not advisable to use *dl*-glutamic acid as a standard for determinations on natural materials.

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[1] Hac, L., private communication.

It was found that α-ketoglutaric acid can replace glutamic acid for the growth of *Lactobacillus arabinosus*, if it is present in relatively high concentrations. At low concentrations this substance was completely inactive.

TABLE I

Comparison of Activity of dl- and l(+)-Glutamic Acid for Growth of Lactobacillus arabinosus

dl-Glutamic acid tested	Assay value, *l*(+)-glutamic acid used as standard	Activity ratio *dl*:*l*(+)
mg.	*mg.*	
0.02	0.0102	0.51
0.04	0.021	0.53
0.12	0.066	0.55
0.16	0.096	0.60
0.20	0.130	0.65
0.24	0.180	0.75

TABLE II

Glutamic Acid Activity of α-Ketoglutaric Acid

l(+)-Glutamic acid	α-Ketoglutaric acid	Titration values,* 0.1 N NaOH	
mg.	*mg*	*ml*	*ml.*
0	0	0.51	0.55
0.02	0	1.01	1.05
0.04	0	1.73	1.68
0	0.02	0.53	0.55
0	0.04	0.60	0.55
0.05	0	2.05	2.05
0.05	0.04	2.28	2.30
2.00	0	7.50	7.61
0	2.00	5.36	5.55

* 5 ml. aliquots from 10 ml. culture tubes.

In tests in which limiting amounts of glutamic acid were present to initiate growth, the amount of lactic acid produced was increased by the further addition of rather small amounts of α-ketoglutaric acid (Table II).

β-Hydroxyglutamic acid was tested and found to be completely inactive.

Table III shows that pyrrolidonecarboxylic acid cannot replace glutamic acid for the growth of this organism. When it was hydrolyzed by being boiled for 4 hours with 6 N hydrochloric acid, this substance had theoretical glutamic acid activity.

It is well known that, when very slightly acid solutions of glutamic acid are heated, the glutamic acid is converted to pyrrolidonecarboxylic acid at a rate which depends on the pH. The system has been studied in detail by Wilson and Cannan (12). In the tests shown in Table IV, pure glutamic acid was dissolved in distilled water and no buffer was added. The autoclaving was done at 15 pounds pressure. Practically all of the activity which was lost by heating the solution was restored by boiling for 4 hours with 6 N hydrochloric acid. Under conditions such as these, the conversion of glutamic acid to pyrrolidonecarboxylic acid is apparently not accompanied by racemization.

TABLE III

Glutamic Acid Activity of Pyrrolidonecarboxylic Acid

Substance tested	Amount	Calculated *dl*-glutamic acid equivalent	Titration value,* 0.1 N NaOH	
	mg.	*mg.*	*ml.*	*ml.*
dl-Glutamic acid	0		1.30	1.32
	0.04		2.08	2.05
	0.08		2.75	2.78
	0.12		3.35	3.30
	0.16		3.91	3.88
Pyrrolidonecarboxylic acid (prepared from *dl*-glutamic acid)	0.0351	0.04	1.31	1.34
	0.105	0.12	1.33	1.32
	0.439	0.50	1.32	1.34
Pyrrolidonecarboxylic acid + *dl*-Glutamic acid	0.105 0.12		3.35	3.36
Hydrolyzed pyrrolidonecarboxylic acid	0.0351	0.04	2.08	2.10
	0.105	0.08	2.80	2.80
	0.439	0.16	3.95	3.95

* 5 ml. aliquots from 10 ml. cultures.

TABLE IV

Effect of Prolonged Heating on Activity of Glutamic Acid Solutions

Concentration of glutamic acid, 1 mg. per 10 ml. of solution

Autoclaving time	*l*(+)-Glutamic acid, as determined by microbiological analysis
hrs.	*mg.*
0	1.00
1	0.76
3	0.47
6	0.19
6 (Followed by hydrolysis with 6 N HCl)	0.98

The titration values of the glutamic acid tests were found to be modified by large variations in the amount of aspartic acid contained in the medium. The medium as used in this investigation contained 4 mg. of aspartic acid per tube. Tests showed that the further addition of the small amounts of aspartic acid contained in the materials to be tested was without effect on the titration values.

Glutamic Acid Content of Peptides, Proteins, and Foodstuffs

Complete hydrolysis of peptides and proteins was found to be essential for the accurate determination of glutamic acid by the method reported here. A casein sample peptonized by heating with dilute acid showed a glutamic acid content of 18.2 per cent as compared to a value of 21.5 per cent obtained after hydrolysis with 6 N hydrochloric acid. Consistently higher values were obtained when proteins and foodstuffs were hydrolyzed with hydrochloric acid than when sulfuric acid hydrolysis followed by removal of the sulfate by barium hydroxide was used. The lower values in the latter case were probably due to adsorption of glutamic acid on the barium sulfate precipitates.

TABLE V

Effect of Hydrolysis on Determination of Glutamic Acid in Glutathione

Theoretical $l(+)$-glutamic acid content of sample	$l(+)$-Glutamic acid found			
	Non-hydrolyzed sample		Hydrolyzed sample	
mg	*mg.*	*per cent of theoretical*	*mg.*	*per cent of theoretical*
0 02	0.0100	50	0.0205	102
0.04	0.0155	39	0.0410	102
0.06	0.0210	35	0.0600	100

Samples of pure $l(+)$-glutamic acid which were refluxed for 24 hours with 6 N hydrochloric acid showed no loss in glutamic acid activity.

The analytical procedure was tested by determining the glutamic acid content of pure glutathione. When this peptide was not hydrolyzed, very low results were obtained. After hydrolysis with 6 N hydrochloric acid practically theoretical values were obtained (Table V).

In Tables VI and VII the authors' values for the glutamic acid content of casein and egg albumin are compared with values taken from the literature. It is recognized that the accurate determination of glutamic acid by isolation procedures is exceedingly difficult. Considerable work has been done by Chibnall and associates (13, 17) to make the determination by this procedure as accurate as possible. In the case of egg albumin these authors report that not all of the material which they isolated was $l(+)$-glutamic acid. A small portion was reported as being the racemic compound. This probably accounts for the difference between their values and those obtained by the present authors.

In Table VIII the glutamic acid content of a few foodstuffs is given. The formation of humin during hydrolysis is almost certain to have caused small losses of glutamic acid. The hydrolysis method developed by Holland (18) and associates offers a means of avoiding this error.

TABLE VI

Glutamic Acid Content of Casein

Description of preparation	Investigator	Total nitrogen	Glutamic acid
		per cent	*per cent*
Labco casein	Present authors	15.20	21.5*
Purified by repeated pptn. at isoelectric point	" "	15.62†	22.4*†
" "	Bailey *et al.* (13)	15.73†	22.0†
Purified preparation	Dakin (14)		21.0
	Olcott (15)		22.0

* *l*(+)-Glutamic acid.

† Corrected for ash content.

TABLE VII

Glutamic Acid Content of Egg Albumin

Description of preparation	Investigator	Total nitrogen	Glutamic acid
		per cent	*per cent*
Commercial sample repurified by repeated pptn. with Na_2SO_4	Present authors	15.54*	14.3*†
Crystalline sample prepared from fresh egg	" "	15.56*	14.3*†
Crystalline preparation	Calvery (16)	15.12	14.0
Recrystallized sample prepared by R. K. Cannan	Chibnall *et al.* (17)	15.74*	16.1*
Supplied by D. Breese Jones	Olcott (15)		16.9

* Corrected for ash content.

† *l*(+)-Glutamic acid.

Recovery tests on *l*(+)-glutamic acid added to a variety of hydrolysates of foodstuffs are shown in Table IX. These data do not indicate the presence of any substance in the hydrolysates which interferes with the biological test.

An attempt was made to evaluate the loss of glutamic acid due to humin formation. Casein was hydrolyzed with 6 N hydrochloric acid in the presence of various carbohydrates and the glutamic acid content of the hydrolysates was determined. Table X shows that some loss did occur but the loss was less than might have been expected.

TABLE VIII

l(+)-Glutamic Acid Content of Some Foodstuffs

Material analyzed	Protein* content of undried samples	l(+)-Glutamic acid found		
		Undried basis	Moisture-free basis	Glutamic acid N as per cent of total N
	per cent	*per cent*	*per cent*	
Wheat.	15.57	4.92	5.56	17.2
Brewers' yeast.	54.74	6.07	6.31	6.6
Cottonseed meal. . . .	42.87	7.61	8.31	10.6
Peanut meal.	44.69	7.76	8.59	10.3
Soy bean meal.	44.28	6.83	7.64	9.2
Tankage	62.77	5.91	6.41	5.6

* Kjeldahl N multiplied by the factor 6.25 except for wheat for which the factor 5.7 was used.

TABLE IX

Recovery of Glutamic Acid Added to Hydrolysates

Material analyzed	l(+)-Glutamic acid			
	Found in aliquot of hydrolysate	Added	Total found	Per cent recovery
	mg.	*mg.*	*mg.*	
Wheat	0.0135	0.02	0.0335	100
	0.0590	0.02	0.0790	100
	0.0135	0.04	0.0540	101
	0.0590	0.04	0.1010	105
Cottonseed meal	0.0160	0.02	0.0355	98
	0.0610	0.02	0.0800	95
	0.0160	0.04	0.0550	98
	0.0610	0.04	0.1020	102
Tankage	0.0205	0.02	0.0410	102
	0.0395	0.02	0.0605	105
	0.0205	0.04	0.0600	99
	0.0395	0.04	0.0810	104
Yeast	0.0180	0.02	0.0390	105
	0.0355	0.02	0.0555	100
	0.0180	0.04	0.0590	102
	0.0355	0.04	0.0745	98

DISCUSSION

The question of the possible racemization of glutamic acid by boiling hydrochloric acid is of importance in connection with the microbiological determination of glutamic acid by the use of *Lactobacillus arabinosus* since the $d(-)$ isomer is much less active than the $l(+)$ form for the growth of this organism. Arnow and Opsahl (19) have reported that a small amount of racemization took place in their experiments in which $l(+)$-glutamic acid was boiled with 20 per cent hydrochloric acid. After a period of 30 hours 2.4 per cent of the original sample had been converted into the *d* isomer. For a period of 24 hours this would be less than 2 per cent, an amount which would come within the over-all experimental error of the microbiological method. In experiments by the present authors, the refluxing of $l(+)$-glutamic acid with 6 N hydrochloric acid for 24 hours did not result in a measurable loss of glutamic acid activity.

Although α-ketoglutaric acid can partially replace glutamic acid in the nutrition of *Lactobacillus arabinosus*, the α-keto analogue is much less active than glutamic acid. It is not probable that hydrolysates of foodstuffs would contain sufficient quantities of this substance to interfere with the glutamic acid determinations.

TABLE X

Effect of Hydrolyzing Casein in Presence of Carbohydrates on Determination of Glutamic Acid

Carbohydrate added to 1 gm casein* before hydrolysis	$l(+)$-Glutamic acid found
	per cent
None	21.5
Sucrose, 1 gm.	20.8
Arabinose, 1 gm	20.8
Soluble starch, Merck, 5 gm.	20.1

* Labco casein was used in these tests.

If glutamine or pyrrolidonecarboxylic acid is present in materials to be analyzed, these substances would be converted to glutamic acid by acid hydrolysis and determined as glutamic acid by this or any other method.

SUMMARY

A microbiological method for the determination of $l(+)$-glutamic acid with *Lactobacillus arabinosus* 17-5 as the test organism is described. The method is applicable to the determination of glutamic acid in peptides, proteins, and foodstuffs. The validity of the method has been tested by the analysis of pure proteins of known glutamic acid content.

The relationship of glutamine and glutamic acid to the growth requirements of *Lactobacillus arabinosus* has been studied. The data suggest that glutamine must be formed from glutamic acid by the organism if it is not present in the medium and that the organism cannot readily carry out this conversion when the glutamic acid concentration is low.

Tests on the specificity of the organism for glutamic acid showed that glutamine is very active, that α-ketoglutaric acid is active under certain conditions, and that β-hydroxyglutamic acid and pyrrolidonecarboxylic acid are completely inactive. The ratio of the activity of *dl*- and *l*(+)-glutamic acid was found to vary, depending on the test level.

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BIBLIOGRAPHY

1. Kuiken, K. A , Norman, W. H , Lyman, C M., Hale, F., and Blotter, L , *J. Biol Chem.*, **151**, 615 (1943)
2. Kuiken, K A , Norman, W. H., Lyman, C. M , Hale, F , and Blotter, L., *Science*, **98**, 266 (1943).
3. Pollack, M A , and Lindner, M , *J Biol. Chem.*, **143**, 655 (1942).
4. Shankman, S , *J Biol Chem* , **150**, 305 (1943)
5. Hegsted, D. M., *J Biol Chem* , **152**, 193 (1944)
6. Shankman, S , Dunn, M S , and Rubin, L B , *J. Biol Chem* , **150**, 477 (1943).
7. Van Slyke, L L , and Baker, J C , *J Biol Chem.*, **35**, 127 (1918)
8. Cohn, E J., *J Gen Physiol* , **4**, 697 (1922)
9. Cohn, E J , and Hendry, J. L , *J Gen. Physiol.*, **5**, 521 (1923).
10. Kekwick, R A , and Cannan, R. K., *Biochem. J.*, **30**, 277 (1936).
11. Chibnall, A C , Rees, M W , and Williams, E. F., *Biochem. J* , **37**, 354 (1943).
12. Wilson, H , and Cannan, R K., *J. Biol. Chem.*, **119**, 309 (1937).
13. Bailey, K., Chibnall, A C., Rees, M W., and Williams, E. F., *Biochem. J.*, **37**, 360 (1943).
14. Dakin, H D , *Biochem J.*, **12**, 290 (1918)
15. Olcott, H S , *J. Biol Chem* , **153**, 71 (1944).
16. Calvery, H O , *J Biol Chem* , **94**, 613 (1931–32)
17. Chibnall, A C , Rees, M W , and Williams, E. F., *Biochem. J.*, **37**, 372 (1943).
18. Holland, B. R., Lyman, C M., and Hale, F., Abstracts, 108th meeting of the American Chemical Society, New York, 24B (1944).
19. Arnow, L. E., and Opsahl, J. C., *J. Biol. Chem.*, **133**, 765 (1940).

THE FECAL EXCRETION OF ESTROGENS BY PREGNANT COWS*

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Recent reports (1) that administered estrogen is removed from the circulation and concentrated in the bile suggest the possibility that considerable quantities of estrogen may leave the body by alimentary elimination.

Although many investigations of the urinary excretion of estrogens have been reported, only three reports of the estimation of the fecal excretion of these substances have been found. Dohrn and Faure (2) reported that pregnant women excrete large quantities of estrogenic material with the feces. Siebke and Schuschania (3) stated that normal women excrete almost as much estrogen in the feces as in the urine. Gustavson (4) found large amounts of estrogenic activity in the feces (plus urine?) of hens. A systematic study of the quantity and chemical nature of the fecal estrogens has apparently never been made.

An investigation of fecal excretion of estrogenic substances by pregnant cows was therefore undertaken. Cows were chosen because apparently this species excretes but little estrogen in the urine. Although the amounts of estrogen found in the feces are not large, the behavior of the material indicates that the active substance is neither estrone nor estriol. On the basis of chemical separation, distribution behavior, and activity ratios as determined in rats and mice, the conclusion has been reached that the active substance obtained from the feces of pregnant cows is largely a non-ketonic weakly acidic phenol, probably α-estradiol.

Whether estradiol is secreted as such into the gut by the cow is not known. It is possible that the presence of estradiol in the feces is, rather, due to an intraintestinal conversion of some other estrogen (estrone?) into estradiol. Mamoli (5) has shown that certain strains of yeast, when incubated with estrone esters, reduce the estrone to estradiol and simultaneously hydrolyze the ester linkage. Likewise, Mamoli (6) has shown that *Bacillus putrificus*, isolated from putrifying tissue extracts, has the ability to reduce ketonic groups and unsaturated linkages of certain androgenic steroids. It seems not improbable, therefore, that some component (or components) of the intestinal flora of the cow may have a similar capacity

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for reduction of steroid ketonic groups. On the other hand, Westerfeld and Doisy (7) have reported that the estrogen of cow ovaries is entirely non-ketonic. This raises the distinct possibility that in this species, at least, estradiol as such enters the gut from the blood stream or with the bile.

EXPERIMENTAL

Feces from cows during the last few weeks of pregnancy were collected without contamination with urine.[1] The fresh feces were thoroughly extracted with ethyl alcohol, either at room temperature or in large Soxhlet extractors. The yields of active material appeared to be independent of extraction temperature. Hydrolytic procedures were not used because in a number of pilot experiments no evidence for the presence of conjugated estrogen could be obtained.

TABLE I

Estrogen Content of Pregnant Cow Feces

Preparation No.	Cow No.	Feces collected		Fresh feces			Dried feces	
		After breeding	Before calving	Solids	Estrogen per kilo solids		Estrogen per kilo solids	
		days	*days*	*per cent*	*rat units*	γ*	*rat units*	γ*
L670	H38		1	13.2	5000	900	4000	720
L675	492		12	13.6	5000	900	2500	450
L677	150X	274	5	13.1	7000	1260	2700	486
715	390X	277	3	13.2	7500	1350		
719	H36 H53 G23 491	244–258	24–38	14.8	8000	1440	2700	486

* Expressed as micrograms of α-estradiol calculated on the basis of 0.18 γ of estradiol per rat unit. By our method of assay, using α-estradiol in sesame oil solution and administering the entire dose in one injection, 39 per cent of the animals respond to 0.15 γ, 70 per cent to 0.18 γ, and 84 per cent to 0.21 γ.

The combined alcoholic extracts were distilled *in vacuo* to an aqueous sludge which was then acidified to Congo red with hydrochloric acid and exhaustively extracted with ethyl ether. Strongly acidic substances were removed from the combined ethereal solutions by extraction with 3 per cent $NaHCO_3$ solution. After washing the ether solutions with water till neutral to litmus, the ether was distilled off and an aliquot of the resulting residue was used for assay purposes. The yields of estrogen obtained from five specimens of pregnant cow feces are given in Table I.

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[1] The author is greatly indebted to Dr. Ralph Reece of the New Jersey Agricultural Experiment Station for his wholehearted cooperation in arranging for the collection of the feces.

The remainder of the crude tarry material was freed of neutral substances by being dissolved in a small volume of ether and exhaustively extracted with 1 N NaOH. The alkaline solution was acidified and active phenols transferred back to ether by extraction with this solvent. The ether was distilled off and the resulting oil was dried *in vacuo* over $CaCl_2$.

The dry oil was separated into ketonic and non-ketonic fractions by means of Girard's Reagent T (8), by means of a slight modification of the method described by Pincus and Pearlman (9). Since almost all the activity was found in the non-ketonic fraction (Table II), the latter was further divided into strongly and weakly phenolic fractions by partition between benzene and 0.3 M Na_2CO_3, as described by Mather (10). As shown by the distribution ratios in Table II, almost all of the activity of the non-ketonic extract was found in the weakly phenolic fraction. The chemical separation described above therefore shows that a major proportion of the estrogenic activity present in feces of pregnant cows is due to a non-ketonic, weakly acidic phenol. Of the known estrogens, only estradiol fulfils this description.

TABLE II

Distribution of Estrogen of Pregnant Cow Feces

Feces	Preparation No	Neutral	Phenolic		
			Ketonic	Non-ketonic	
				"Strong"	"Weak"
		per cent	*per cent*	*per cent*	*per cent*
Fresh	715 → 723C	2	5.8	4.0	88.2
	719 → 730B	3	6.8	17.5	72.7
	Pooled → 712B	0	4.5	0	95.5
Dried*	670 → 707	0	55	45	
	675 → 709	0			
	677 → 725	0	67	33	

* The dried samples were aliquots of the fresh samples.

Further evidence concerning the identity of an estrogenic substance may be obtained by study of the response of rats and mice to administration of the substance. Doisy *et al.* (11) have shown that the ratio of the weight of the rat unit to that of the mouse unit is quite distinct for each of the more common estrogens, *e.g.* estrone, estriol, and estradiol. Our own assay data closely approximate those obtained in Doisy's laboratory.

Furthermore, by assaying oily as well as aqueous solutions and comparing the rat unit obtained with each type of vehicle, the ratios obtained clearly distinguish estriol from estrone and α-estradiol, although the latter two cannot be distinguished from each other in this way.

In order to confirm the indications of partition behavior regarding the nature of the active principle of pregnant cow feces, the final non-ketonic weakly acidic phenol fraction was assayed in spayed mice and rats. In the latter species both oily and aqueous solutions were assayed. The precautions used in preparing the assay animals and the assay technique closely followed those outlined by Kahnt and Doisy (12), except that the solutions in sesame oil were administered in a single dose while the total dose of aqueous (10 per cent ethanol) solutions was divided into two injections given 8 hours apart. Vaginal smears were made beginning 48 hours after the first injection. The results obtained with pure estrone, estriol, and α-estradiol,[2] as well as with several specimens of the non-ketonic weakly acidic phenol fraction of pregnant cow feces, are detailed in Table III. The ratio of the rat unit obtained with sesame oil solutions to that obtained with aqueous solutions (1.27:1 to 1.60:1) indicates that the active constituent of the extracts is definitely not estriol (ratio 16:1) but may be either estradiol (ratio 1.5:1) or estrone (ratio 1.35:1). Likewise the ratio of the weight of the rat unit to that of the mouse unit (aqueous solutions) obtained with the extracts (between 3.33:1 and 4.0:1) agrees closely with that obtained with pure α-estradiol (4.0:1) but does not correspond to the ratios for estrone or estriol (14:1 and 0.08:1, respectively). The chemical and physiological data therefore agree in indicating that all or nearly all of the estrogen excreted with the feces by cows late in pregnancy is estradiol.

TABLE III

Comparison of Assay Behavior of Weakly Phenolic, Non-Ketonic Fraction from Pregnant Cow Feces with That of Crystalline Estrogens

Preparation	Rat unit		Mouse unit, aqueous	Rat unit, oil / Rat unit, aqueous	Rat unit, aqueous / Mouse unit, aqueous
	Oil	Aqueous			
	γ	γ	γ		
Estrone	1.35	1.00	0.07	1.35:1	14:1
α-Estradiol	0.18	0.12	0.03	1.5:1	4.0:1
Estriol	±32	2.0	>24	16:1	0.08:1
	ml	*ml*	*ml*		
L712B*	0.14	0.11	0.03	1.27:1	3.67:1
L723C*	0.023	0.016	0.004	1.44:1	4.00:1
L730B*	0.032	0.020	0.006	1.60:1	3.33:1

* The units of the unknown substance are expressed in terms of ml. of standard alcoholic solution.

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[2] The crystalline estrogens were kindly furnished by Dr. Oliver Kamm of Parke, Davis and Company, and by Dr. Erwin Schwenk of the Schering Corporation.

A perplexing finding is that illustrated by the data in the second half of Table II. Extracts were made from three specimens of feces which had been oven-dried at 70–80°. The dried feces were extracted with warm ethanol in Soxhlet extractors and the extracts were then treated as described above. On separating the purified phenolic fractions with Girard's reagent, it was found that one-half to two-thirds of the estrogenic activity was present in the ketonic fractions. Repetition of the Girard's separation confirmed the ketonic nature of this fraction. The samples of dried feces used in these experiments were aliquots of the samples extracted in the fresh state as described above. The drying was begun at the same time that the fresh aliquots were extracted. It, therefore, appears that as a result of drying the fecal specimens in air at least a portion of the estradiol is converted to a ketonic estrogen, presumably estrone. Coincidentally, a considerable proportion of the extractable active material is lost.

The technical assistance of Miss Dorothy Wangerin in performing the assays is gratefully acknowledged.

SUMMARY

During the last 2 weeks of pregnancy, cows excrete 5000 to 10,000 rat units of estrogenic substance per kilo of dry feces. Calculated as α-estradiol, this amounts to 0.9 to 1.4 mg. of estradiol per kilo of fecal solids.

The fact that the major proportion (73 to 96 per cent) of the estrogenic activity is found in the weakly phenolic, non-ketonic fraction of the extracts strongly indicates that the active substance is estradiol. Likewise, the ratio of the weight of the rat unit to that of the mouse unit is in good agreement with the similar ratio obtained with pure α-estradiol, but not with that of estrone or estriol.

BIBLIOGRAPHY

1. Cantarow, A., Rakoff, A E , Paschkis, K. E., and Hansen, L. P., *Proc. Soc. Exp. Biol. and Med.*, **49**, 707 (1942). Cantarow, A , Rakoff, A. E., Paschkis, K. E., Hansen, L. P., and Walking, A. A., *Endocrinology*, **31**, 515 (1942); *Proc. Soc. Exp. Biol. and Med.*, **52**, 256 (1943).
2. Dohrn, M., and Faure, W., *Klin. Wochschr.*, **7**, 943 (1928).
3. Siebke, H., and Schuschania, P., *Zentr. Gynäk.*, **54**, 1734 (1930).
4. Gustavson, R. G., *Proc. Am. Soc. Biol Chem.*, *J. Biol. Chem.*, **92**, p. lxxi (1931).
5. Mamoli, L., *Ber. chem. Ges.*, **71**, 2696 (1938).
6. Mamoli, L., Koch, R., and Teschen, H., *Z. physiol. Chem.*, **261**, 287 (1939).
7. Westerfeld, W. W., and Doisy, E. A., *Ann. Int. Med.*, **11**, 267 (1937).
8. Girard, A., and Sandulesco, G., *Helv. chim. acta*, **19**, 1095 (1936).
9. Pincus, G., and Pearlman, W. H., *Endocrinology*, **29**, 413 (1941).
10. Mather, A., *J. Biol. Chem.*, **144**, 617 (1942).
11. Westerfeld, W. W., Thayer, S. A., MacCorquodale, D. W., and Doisy, E. A., *J. Biol. Chem.*, **126**, 181 (1938).
12. Kahnt, L. C., and Doisy, E. A., *Endocrinology*, **12**, 760 (1928).

LETTERS TO THE EDITORS

ON THE ENZYMIC FORMATION OF VITAMIN B_c FROM ITS CONJUGATE

Sirs:

Briggs *et al.*[1] have questioned the enzymic character of the conversion of vitamin B_c conjugate to vitamin B_c as reported by Binkley *et al.*[2] The enzyme (or enzymes) involved is widely distributed in nature. Hog kidney, liver, small intestine, and beef liver are rich sources. It occurs in sweet almond and to a lesser extent in potatoes. Only traces were detected in molds (*Aspergillus*, *Penicillium*, and *Mucor*). It does not occur in yeast. For convenience we refer to the enzyme tentatively as vitamin B_c conjugase.

In natural crude mixtures the enzyme is stable on storage. The activity of acetone-desiccated hog kidney was found constant over a period of 2 years and commercial almond meal is uniformly active. Solutions of purified conjugase from hog kidney and from almond are stable for several weeks in the refrigerator but dried preparations lose activity on standing. The enzyme is destroyed by boiling for 2 minutes. Kidney and liver autolysates are active. We have been unable to identify vitamin B_c conjugase with kidney nucleosidase,[3] acid phosphatase of almond[4] or potato,[5] alkaline phosphatase of small intestine,[6] or β-glucosidase of almond.[4] The optimum pH of the kidney enzyme is 4.5, while that for almond is 7.0.

The following experiment is illustrative. A frozen hog kidney (118 gm.) was dispersed in water (354 cc.). The mixture was incubated under toluene for 40 hours at 37°. It was centrifuged and filtered (Super-Cel). To the filtrate (320 cc.) was added ammonium sulfate (96 gm.), and the precipitate collected at the centrifuge. The precipitate was taken up in water (75 cc.). Acetic acid was added to pH 4.1. The precipitate was centrifuged off and discarded. Ammonium sulfate (21.0 gm.) was added to the solution (70 cc.) and the centrifuged precipitate was suspended in water (26 cc.). The suspension was centrifuged and the insoluble fraction discarded. The clear solution was adjusted to pH 6.5 with dilute NaOH and stored in the refrigerator under toluene. It contained 354 mg. of protein.

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[1] Briggs, G M., Jr., Luckey, T. D., Elvehjem, C. A , and Hart, E B., *J. Biol. Chem.*, **155**, 687 (1944).

[2] Binkley, S. B., Bird, O. D , Bloom, E. S., Brown, R. A , Calkins, D G , Campbell, C. J., Emmett, A. D., and Pfiffner, J. J., *Science*, **100**, 36 (1944).

[3] Levene, P. A., Yamagawa, M., and Weber, I., *J. Biol. Chem* , **60**, 693 (1924). Levene, P. A., and Weber, I., *J. Biol. Chem.*, **60**, 707 (1924). von Euler, H., and Brunius, E., *Ber. chem. Ges.*, **60**, 1584 (1927).

[4] Brederick, H., *Ber. chem. Ges.*, **71**, 410 (1938).

[5] Hartmann, M., and Bosshard, W., *Helv. chim. acta*, **21**, 1554 (1938).

[6] Schmidt, G., and Thannhauser, S. J., *J. Biol. Chem.*, **149**, 369 (1943).

To 0.25 cc. of the solution were added 1 cc. of substrate (a yeast concentrate having in each cc. 60 γ of vitamin B_c as conjugate), 3.75 cc. of distilled water, and 5 cc. of 0.1 N sodium acetate buffer, pH 4.5. The mixture was incubated at 45° in a water bath. Aliquots were withdrawn, heated in a boiling water bath, and assayed microbiologically (*Streptococcus lactis* R) with the following results:

Time *hrs.*	Vitamin B_c liberated per mg protein γ
0.5	0.43
1	0.79
2	2.02
4	3.99
Boiled enzyme control, 4 hrs.	0.04

Mims *et al.*[7] report an enzyme preparation from rat liver which produces a *Streptococcus lactis* R-stimulating factor from inactive material in yeast. Laskowski and Mims[8] have succeeded in further purifying the enzyme. We have found their purified enzyme (supplied by Dr. Laskowski) active in forming vitamin B_c from vitamin B_c conjugate.

These observations demonstrate that the reaction is enzymic in nature.

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[7] Mims, V., Totter, J. R., and Day, P. L., *J. Biol. Chem.*, **155**, 401 (1944).

[8] Laskowski, M., and Mims, V., personal communication.

THE RÔLE OF DIHYDROCOZYMASE IN ENZYMATIC TRANSPHOSPHORYLATION

Sirs:

Aqueous extracts of minced rat muscle can be shown to contain the enzymes which catalyze the following two reactions.

(1) Adenosine triphosphate + glucose → glucose-6-phosphate + adenosine diphosphate
(2) Adenosine triphosphate + fructose-6-phosphate → fructose-1,6-diphosphate + adenosine diphosphate

Both of these reactions can be followed manometrically in fluoride-inhibited extracts by a method described previously[1] for measuring the activity of yeast hexokinase, which catalyzes Reaction 1.

When the muscle extracts are acidified to pH 6 and kept at 25°, there is a very rapid disappearance of the activity of both enzymes. This loss of activity is much slower at pH 6.5 and does not occur at all in the pH range 7.0 to 8.0. When the water extracts are prepared from acetone-dried rat muscle instead of from fresh muscle, the enzymes are stable at any pH in the range 6.0 to 8.0. An extract of acetone-dried muscle can, however, be inactivated rapidly at pH 6.0 by the addition of an extract of fresh muscle.

Extracts which have been inactivated at pH 6.0 can be reactivated by the addition of small amounts of dihydrocozymase, as shown in the table, in which the manometric data have been substantiated by chemical estimation of the disappearance of adenosine triphosphate. The results were obtained with a fluoride-iodoacetate-inhibited system, in which the addition of the oxidized form of cozymase[2] had practically no effect. It seems clear that the effect of dihydrocozymase observed here is not related to its well known action in hydrogen transport, since no oxidation-reduction could have taken place. Dihydrocozymase appears to function directly in the reactions by which phosphate is transferred from adenosine triphosphate to glucose or fructose-6-phosphate.

This fact suggests a relationship to the Pasteur effect. The concentration of reduced cozymase might be expected to be much lower under aerobic than anaerobic conditions, and it follows that Reactions 1 and 2 would occur at a slower rate under aerobic conditions.

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[1] Colowick, S. P., and Kalckar, H. M., *J. Biol. Chem.*, **148**, 117 (1943).

[2] The cozymase preparation (50 to 75 per cent pure) was generously supplied by Dr. Fritz Schlenk. The dihydrocozymase was prepared by reduction with dithionite according to D. E. Green and J. G. Dewan (*Biochem. J.*, **31**, 1069 (1937)).

Effect of Dihydrocozymase on Transfer of Phosphate from Adenosine Triphosphate to Glucose and Fructose-6-phosphate

Minced rat muscle extracted with 1.4 volumes of H_2O; extract kept at 25° at pH 6.0 for 2.5 hours. The complete test system (volume 2.4 cc.) was as follows: 0.9 cc. of aged extract, 0.025 M $NaHCO_3$, 0.008 M $MgCl_2$, 0.002 M iodoacetate, 0.06 M NaF, 0.0001 M cozymase (oxidized or reduced form), 0.004 M adenosine triphosphate, and 0.01 M phosphate acceptor. Gas phase, 95 per cent N_2-5 per cent CO_2; pH 7.5; reaction time 35 minutes. Trichloroacetic acid filtrates analyzed for inorganic phosphate before (P_0) and after (P_7) hydrolysis for 7 minutes in 1.0 N HCl at 100°. All values are expressed in micromoles.

Sample No	Cozymase addition	Phosphate acceptor	P_0	P_7	P_7-P_0	P transferred	Acid production (manometric)
1	None	None	17.7	30.3	12.6	0	2.9
2	"	Glucose	17.1	29.6	12.5	0.1	3.0
3	"	Fructose-6-phosphate	17.4	30.3	12.9	0	6.5
4	Cozymase	None	16.8	29.6	12.8	0	1.8
5	"	Glucose	17.7	31.0	13.3	0	3.0
6	"	Fructose-6-phosphate	17.7	31.6	13.9	0	6.1
7	Dihydrocozymase	None	18.4	32.2	13.8	0	2.8
8	"	Glucose	17.4	26.1	8.7	6.8*	6.7
9	"	Fructose-6-phosphate	17.7	20.6	2.9	13.8*	14.8

* Corrected for hydrolysis of fructose-1,6-diphosphate (25 per cent in 7 minutes in 1.0 N HCl at 100°).

It is a pleasure to thank Professor C. F. Cori for his continued interest in this work.

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CHROMATOGRAPHIC SEPARATION OF THE 2,4-DINITROPHENYLHYDRAZONES OF ESTRONE AND EQUILENIN

Sirs:

When the 2,4-dinitrophenylhydrazones of estrone and equilenin in benzene solution are passed through a column of aluminum oxide (Brockmann), 5 × 100 mm., they are strongly adsorbed in a yellow band at the top of the column. Elution of the column with 1 per cent ethyl alcohol in petroleum ether causes development of the chromatogram and the hydrazone of estrone separates as a band immediately below the hydrazone of equilenin. Continued washing of the column with 1 per cent ethyl alcohol in petroleum ether removes the hydrazone of estrone but does not remove the hydrazone of equilenin. With columns of aluminum oxide 5 × 100 mm., quantities of estrone hydrazone from 5 to 25 γ are readily separated from approximately 1000 γ of the hydrazone of equilenin in this fashion. The hydrazone of equilenin may be removed from the column by increasing the concentration of alcohol or by washing with acetone. The results, in micrograms, of several such separations are as follows:

Estrone hydrazone	Equilenin hydrazone (approximately)	Estrone hydrazone recovered*
5.1	1000	5.1
25.5	1000	25.5

* Determined by a method of Veitch and Milone (*J. Biol. Chem.*, in press).

Preparation of 2,4-Dinitrophenylhydrazone of Equilenin[1]—Approximately 50 mg. of pure crystalline equilenin were dissolved in 10 ml. of aldehyde-free ethyl alcohol and twice the calculated amount of 2,4-dinitrophenylhydrazine added. The mixture was refluxed for 1 hour, at the end of which time sufficient concentrated hydrochloric acid was added to change the color of the solution from a deep red to a light yellow. At this stage the hydrazone of equilenin partially crystallized from solution. A small amount of water (5 ml.) was added and the mixture cooled to 0° in an ice bath. The orange-colored crystalline hydrazone was filtered off, recrystallized from alcohol-water several times, and finally dried in a vacuum desiccator.

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[1] We are indebted to Dr. Oliver Kamm of Parke, Davis and Company who supplied the pure equilenin used in this work.

M.p. 268-270° (uncorrected) with decomposition. Nitrogen, by Dumas method, found 12.5, theory 12.55.

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CHOLINE-LIKE NUTRITIONAL EFFECT OF DIMETHYLAMINOETHANOL

Sirs:

It was found[1, 2] that supplements of betaine, methionine, or methionine plus aminoethanol did not alleviate the symptoms of choline deficiency in chicks receiving simplified diets which were deficient in choline. Stetten[3] in experiments with rats, observed that ethanolamine could function as a precursor for the biological synthesis of choline. He postulated that betaine might be a source of methyl groups for this synthesis, and subsequently experimental evidence has appeared[4] which supports this hypothesis. It had previously been shown[5] that methionine could act as a donor of methyl groups in the synthesis of choline in the body of the rat and that the addition of betaine enabled rats to utilize homocystine in the diet in place of methionine.[6] Recently it was reported[7] that betaine or methionine when added to a diet containing the natural foods corn and peanut meal apparently enabled the chick to synthesize choline. This effect was not observed when the diet was based principally on glucose and casein.[1, 2, 7] From this it appeared that, in natural foods, simple precursors of choline might exist which could be "methylated" by the chick to yield choline. The possibility of the existence of such precursors prompted a search among various compounds.

In the present investigation, day-old New Hampshire red chicks were placed on a choline-deficient diet similar to that described previously.[2] The diet contained 18 per cent of casein, which is a fairly good source of methionine. Ten chicks were used in each group. The chicks were weighed and examined for perosis at frequent intervals. The results shown in the accompanying table were obtained.

[1] Jukes, T. H., *J. Nutr.*, **22**, 315 (1941). Almquist, H. J., and Grau, C. R., *J. Nutr.*, **27**, 263 (1944).

[2] Jukes, T. H., and Welch, A. D., *J. Biol. Chem.*, **146**, 19 (1942).

[3] Stetten, D., Jr., *J. Biol. Chem.*, **140**, 143 (1941).

[4] Simmonds, S., Cohn, M., Chandler, J. P., and du Vigneaud, V., Abstracts, Division of Biological Chemistry, American Chemical Society, New York, 49B (1944).

[5] du Vigneaud, V., Chandler, J. P., Cohn, M., and Brown, G. B., *J. Biol. Chem.*, **134**, 787 (1940).

[6] du Vigneaud, V., Chandler, J. P., Moyer, A. W., and Keppel, D. M., *J. Biol. Chem.*, **131**, 57 (1939).

[7] McGinnis, J., Norris, L. C , and Heuser, G. F., *Proc. Soc. Exp. Biol. and Med.*, **56**, 197 (1944).

Supplement to 100 gm basal diet	Per cent incidence of perosis at		Gain in 28 days
	21 days	28 days	
			gm.
None	50	60	112
0.2 gm. dimethylaminoethanol	0	0	145
0.1 " choline chloride	0	0	194

The results indicate that dimethylaminoethanol ($(CH_3)_2NCH_2CH_2OH$) has a choline-like effect in promoting growth and preventing perosis in chicks on a diet deficient in choline. The promotion of growth was not as marked as the prevention of perosis, which was complete. The structure of the molecule of dimethylaminoethanol is such that the addition of a methyl group might lead to the formation of choline. The methionine present in the basal diet might function as a source of methyl groups for this purpose. Dimethylaminoethanol occurs in nature in combined form in cassaine, an alkaloid present in a member of the Leguminosae.[8] Dimethylaminoethanol has been reported to have a blood pressure-lowering effect in the dog about one-fifth that of choline.[9] It was reported by du Vigneaud[10] that dimethylaminoethanol could not substitute for choline in effecting growth with homocystine. However, he found that deuteriomethyl groups appeared in choline[4, 10] obtained from the bodies of rats which had been fed with dimethylaminoethanol containing deuterium in the methyl groups. These observations are in harmony with the present suggestion that dimethylaminoethanol may be an acceptor of a methyl group.

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[8] Faltis, F , and Holzinger, J , *Ber. chem. Ges.*, **72**, 1443 (1939).

[9] Fuchs, H., *Z. Biol.*, **81**, 296 (1938–39).

[10] du Vigneaud, V , *Harvey Lectures*, **38**, 39 (1942–43).

THE MODE OF DEAMINATION OF *l*-AMINO ACIDS IN SURVIVING TISSUES

Sirs:

In 1939, Braunstein and Bychkov[1] suggested that oxidative deamination of *l*-amino acids may proceed indirectly, by way of (1) transamination with α-ketoglutaric acid and (2) deamination of the resulting *l*-glutamic acid by its specific dehydrogenase. In support of this scheme, they prepared a cell-free model of *l*-amino acid oxidase from the enzymes and H and NH_2 carriers involved.

We have tested this indirect deamination mechanism in liver and kidney tissues and confirmed it, after some failures, with diluted suspensions of ground kidney (pig, rat).[2]

The following facts indicate that certain *l*-amino acids are actually deaminated in this way.

1. When ground and suspended in saline solutions, cortical kidney tissue fails to deaminate the simpler aliphatic mono- and dicarboxylic amino acids, owing chiefly to dilution and autolytic decomposition of the H and NH_2 carriers.
2. Deamination of *l*-glutamic acid in such preparations is restored to levels observable in intact kidney slices, by addition of cozymase.
3. *l*-Aspartic acid, *l*-alanine, *l*-cysteic acid, *l*-valine, and *l*-leucine are deaminated by diluted suspensions of broken kidney cells with cozymase and α-ketoglutarate, comparable to intact slices. In absence of cozymase or of ketoglutarate, the extent of deamination is negligible. (Due correction must be made for substantial amounts of ammonia formed in cozymase-containing controls without addition of amino acid; disturbing side reactions are suppressed by 0.01 M Na arsenite.) Similar but less clear cut results were obtained with kidney and liver tissues of other animals.
4. The rates of (*a*) deamination of the amino acids mentioned in suspensions supplemented as under (3) and (*b*) deamination in intact kidney slices do not exceed the rates of (*c*) their transamination with ketoglutarate or (*d*) deamination of *l*-glutamic acid. The rates of reactions (*a*), (*b*), and (*c*) decrease in the following similar sequences.

Glutamic acid > aspartic acid > alanine > cysteic acid > valine > leucine

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[1] Braunstein, A. E., and Bychkov, S. M., *Nature*, **144**, 751 (1939); *Biokhymia*, **5**, 261 (1940)

[2] In a recent survey of transamination Cohen mentions that he failed to observe deamination in similar experiments (Cohen, P. P., in A symposium on respiratory enzymes, Madison, 224 (1942)).

5. Besides the aforementioned, and with the possible exception of serine, methionine, and phenylalanine, the natural isomers of which were not available, none of the natural amino acids was found accessible either to transamination or to oxidative deamination in kidney slices or supplemented suspensions.

6. B_1 avitaminosis in rats results in parallel decreases of glutamic aminopherase and "*l*-amino acid oxidase" activities of liver and kidney tissue.[3]

It must be stressed that most amino acids resistant *in vitro* (glycine, lysine, and tryptophane excepted) undergo transformations *in vivo*, leading to amino acids capable of transamination, and may therefore be subject to terminal deamination as outlined above.

The recent discovery by Green *et al.*[4] of specific flavoprotein enzymes, deaminating certain *l*-amino acids and glycine, does not dismiss the suggested indirect deamination, as the substrates most readily attacked by Green's enzymes are poor substrates with tissue slices or suspensions, and vice versa.

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[3] Kritzmann, M G., *Biokhimya*, **8**, 85 (1943).

[4] Green, D. E., Nocito, V., and Ratner, S., *J. Biol. Chem.*, **148**, 461 (1943). Ratner, S., Nocito, V., and Green, D. E., *J. Biol. Chem.*, **152**, 119 (1944)

THE SUCCESSFUL TREATMENT OF VITAMIN M DEFICIENCY IN THE MONKEY WITH HIGHLY PURIFIED LACTOBACILLUS CASEI FACTOR*

Sirs:

In 1935 it was reported that monkeys which were given a diet consisting of refined foodstuffs developed a syndrome characterized by anemia, leucopenia, necrosis of the gums, and diarrhea.[1] It was suggested in 1938 that the deficiency of an unknown substance, for which the term "vitamin M" was proposed,[2] was responsible. The subject has recently been reviewed.[3]

The effectiveness of a highly purified preparation of the *Lactobacillus casei* factor[4] has now been tested in three monkeys rendered cytopenic by the deficient diet (No. 600).[5] Intramuscular injection of 4 or 4.5 mg. of this substance per monkey over a period of a few days results in prompt and complete remission, with return of the total white blood cell and granulocyte counts to normal levels. Even though the monkeys were only moderately anemic, the treatment was followed by crises of 109 and 78 (normal range 2 to 3) reticulocytes per 1000 red blood cells on the 4th day. The erythrocytes more slowly returned to normal levels. The third monkey (No. 131) showed a similar response.

Monkey 145 was very ill when treatment was started. In addition to the cytopenia it showed loss of appetite, mild necrosis of the gums, and a bloody diarrhea. The treatment was followed by rapid improvement in clinical condition; the appetite promptly returned to normal, the gum necrosis cleared, and the stools became normal.

Hutchings *et al.*[4] found that the preparation used in this study is highly active in stimulating the growth of *Lactobacillus casei*, but only slightly effective in stimulating *Streptococcus lactis* R. We have found that treat-

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[1] Day, P. L., Langston, W. C., and Shukers, C. F., *J. Nutr.*, **9**, 637 (1935).

[2] Langston, W. C., Darby, W. J., Shukers, C. F., and Day, P. L., *J. Exp. Med.*, **68**, 923 (1938).

[3] Day, P. L., Vitamins and hormones, New York, **2**, 71 (1944).

[4] Hutchings, B. L., Stokstad, E. L. R., Bohonos, N., and Slobodkin, N. H., *Science*, **99**, 371 (1944).

[5] Totter, J. R., Shukers, C. F., Kolson, J., Mims, V., and Day, P. L., *J. Biol. Chem.*, **152**, 147 (1944).

Response of Cytopenic Monkeys to Highly Purified Lactobacillus casei Factor

Monkey No	Days on Diet 600*	White blood cells	Granulocytes	Red blood cells	Hemoglobin	Reticulocytes	*L. casei* factor injected
		thousands per c mm	*thousands per c.mm.*	*millions per c.mm.*	*gm per 100 ml.*	*per 1000 r.b c*	
145	1	9.05	2.70	4.56	11.2	0	
	40	11.40	2.96	4.38	11.2	3	
	154	6.15	3.08	4.68		3	
	177	4.10	0.49	3.87	11.2	2	1 mg. on 177th, 178th, and 179th days
	180	11.34	1.26	2.77	7.1	24	
	181	8.76	1.97	2.10	6.9	109	0.5 mg. on 192nd, 193rd, and 194th days
	202	9.85	3.62	4.78	9.7	4	
	233	10.40	4.06	5.20	11.3	8	
147	1	13.8	3.45	3.53	8.5	2	
	69	11.8	1.65	4.71	11.6	2	
	97	5.02	1.90	4.20	11.0	6	
	111	5.90	1.95	4.00	11.0	7	1 mg. on 111th, 112th, 113th, and 115th days
	115	7.00	3.29	4.80	10.8	49	
	118	11.50	7.59	5.00	9.2	78	
	121	9.07	4.44	4.71	11.0	8	

* Both animals received 20 mg. of ascorbic acid and 1 mg. of thiamine chloride daily, and 50 mg. of niacin and 2 mg. of riboflavin three times a week. Monkey 145 received in addition a vitamin mixture, the composition of which is given by Totter *et al.*[5] (foot-note to Table II), and 1 gm. of yeast extract (Difco) daily.

ment of this preparation with the enzyme solution of Mims *et al.*[6] greatly increased activity toward *Streptococcus lactis* R.

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[6] Mims, V., Totter, J. R., and Day, P. L., *J. Biol. Chem.*, **155**, 401 (1944).

VITAMIN B_6 AND TRANSAMINATION

Sirs:

The structures of pyridoxal and pyridoxamine[1] and their reversible interconversion by transamination reactions[2] suggested their possible participation in biological transamination. In pursuing this hypothesis, the following experiments were carried out.

Tissue examined	Incubation time	Group A, vitamin B_6-deficient ration*		Group B, vitamin B_6-deficient ration + pyridoxine (3 γ per gm.)		Group C, Purina fox chow	
		No of animals	Per cent trans-amination†	No of animals	Per cent trans-amination	No of animals	Per cent trans-amination
	min						
Liver	5	4	25.8	3	35.4	4	36.6
	15	4	42.7	3	62.4	4	58.9
Heart	5	4	31.5	3	59.1	4	53.3
	15	4	47.4	3	70.4	3	67.9
Kidney	5	2	16.1	2	37.7	2	31.9
	15	2	31.0	1	57.5	3	49.7
Brain	15	1	41.3	1	58.6	3	59.8

* The ration of Conger and Elvehjem (*J. Biol. Chem.*, **138**, 555 (1941)) was used. Animals of Groups A, B, and C averaged 134, 284, and 307 gm. in weight, respectively.

† Each reaction vessel contained 60 mg. of tissue homogenate in 3.0 ml. of 0.1 M phosphate buffer, 1.0 ml. of 0.06 M glutamic acid, and 0.3 ml. of 0.12 M oxalacetic acid. Final volume, 4.3 ml.; pH 7.4; *T* 38°. The reaction reaches equilibrium at about 75 per cent transamination The figures given are averages of separate samples from four animals. Although there was some variation, there was no overlapping of values between the vitamin B_6-deficient and sufficient groups. With liver tissues, for example, the maximum variations in per cent transamination for samples from the four animals after 15 minutes incubation time were as follows: Group A, 48.3 to 37.4; Group B, 70.3 to 53.9; Group C, 63.5 to 56.7.

Four separate concentrates of transaminase from pig heart prepared by Cohen's procedure[3] were assayed for total vitamin B_6 by the yeast growth method.[4] They contained 12.7, 8.6, 13.3, and 11.5 γ of vitamin B_6 per gm. of protein, expressed as pyridoxine.

Tissues from control and vitamin B_6-deficient rats were examined for their ability to catalyze the reaction, glutamic acid + oxalacetic acid → α-ketoglutaric acid + aspartic acid. Experimental procedures of Cohen[5] were followed. Results (see the table) show a consistent and marked decrease in the ability of tissues from vitamin B_6-deficient rats to catalyze this reaction. Corresponding results were later obtained with a second series of animals.

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[1] Snell, E. E., *J. Biol. Chem.*, **154**, 313 (1944).

[2] Snell, E. E., *J. Am. Chem. Soc.*, in press.

[3] Cohen, P. P., *J. Biol. Chem.*, **136**, 565 (1940).

[4] Atkin, L., Schultz, A. S., Williams, W. L., and Frey, C. N., *Ind. and Eng. Chem., Anal. Ed.*, **15**, 141 (1943).

Attempts to increase the rate of transamination by additions of pyridoxine, pyridoxamine, and pyridoxal with or without adenosine triphosphate to homogenized rat liver from deficient animals gave variable results. Activations ranging from 10 to 50 per cent were obtained in six instances; no activation was observed in three experiments. In the successful experiments, pyridoxal was superior to pyridoxamine as activator, while pyridoxine showed no effect. Addition of adenosine triphosphate was essential for activation.[6] The amounts of activators required were considerably higher than those which occur in normal liver tissue.

These results indicate that vitamin B_6 promotes biological transamination in some manner. Whether it functions directly as cotransaminase, as may reasonably be postulated, or by a more indirect route remains to be determined.

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[5] Cohen, P. P., and Hekhuis, L., *J. Biol. Chem.*, **140**, 711 (1941).

[6] Gunsalus, I. C., Bellamy, W. D., and Umbreit, W. W., *J. Biol. Chem.*, **155**, 685 (1944).

THE INHIBITION OF ARGINASE BY AMINO ACIDS*

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In the course of an attempt to develop a rapid method for the enzymatic determination of arginine it was observed that a quantity of arginase, which would hydrolyze 31 mg. of pure arginine in less than 1 hour, took much longer to achieve the same performance within a protein hydrolysate, 4 hours in the case of arachin, 5 in that of gelatin, and even more in that of casein. The obvious inference was that the action of arginase is inhibited by at least some, and possibly by all, amino acids. The powerful inhibiting effect of *ornithine*, first observed by Gross (1) and since confirmed by others (2–5), would of course come into play even with pure arginine. *Cysteine*, which has been the object of many studies (6–20), appears under some conditions to inhibit, but under others to activate. To the influence of other amino acids upon arginase activity we have found only two references. Edlbacher and Zeller (2) showed that *glycine*, *alanine*, and *lysine* were all inhibitors, the first two relatively feeble, the last nearly as effective as ornithine; and Kitagawa (21), in a paper to which we have not had access, is reported to have observed inhibition by *canaline* and by several other amino acids, the most active of which was *dl-α-aminovaleric acid*. Considering the paucity of available information upon the subject, we have thought it worth while to investigate quantitatively the effect upon arginase activity of a fairly large series of amino acids and of some amino acid derivatives. The results have proved to be not only of practical, but also of considerable theoretical interest.

Materials

Pure *l(+)-arginine hydrochloride* was obtained from the commercial product by conversion into, and recovery from, the flavianate.

l(+)-Ornithine and *l(+)-lysine* were used as their *monohydrochlorides*. The first was prepared by the enzymatic method from gelatin (22); it contained 16.54 per cent N (theory 16.62) and is believed to have been optically pure ($[\alpha]_D = +11.0°$ in 5.5 per cent solution). The second was a commercial product recrystallized once by addition of alcohol (23); it contained 15.2

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* A preliminary account of this work has appeared in abstract in the Proceedings of the Toronto Biochemical and Biophysical Society (*Canad. Chem. and Process Inds.*, **26**, 242 (1942)).

per cent N (theory 15.34) and gave $[\alpha]_D = +9.6°$ in 10 per cent solution. A 3.33 per cent solution containing one additional equivalent of HCl gave for the dihydrochloride (4 per cent concentration) $[\alpha]_D = +15.8°$. Previously reported values, for concentrations of lysine dihydrochloride between 2 and 18 per cent, vary from +14.0° to +16.7° (24–27). The rotation of the monosalt does not appear to have been measured before.

l-Histidine was prepared from its dihydrochloride (28) by precipitation as the diflavianate (29), solution of the latter in dilute ammonia, removal of the flavianic acid with baryta and of excess baryta with H_2SO_4, decolorization with norit, evaporation, and crystallization of the free base from 50 per cent alcohol. The product had $[\alpha]_D = -40.1°$ ($c = 4.4$).

d-Leucine (30), *formyl-l(+)-valine*, and *formyl-dl-valine* (31) were synthetic products, for which we are indebted to Dr. H. O. L. Fischer of the Department of Chemistry. *dl-Valine* was prepared from its formyl derivative by hydrolysis with H_2SO_4. *dl-Serine* (from human hair) was furnished by Dr. J. M. R. Beveridge of the Banting and Best Department of Medical Research. *l(+)-Carbamylarginine*, *l-argininic acid*, and *l(+)-citrulline* were prepared from arginine, the first according to Boon and Robson (32), the second according to Felix and Schneider (33), the third by the procedure of Gornall and Hunter (34). *l(+)-α-Carbamylornithine* was obtained from carbamylarginine by the action of arginase (35). *l(+)-Proline* was prepared from gelatin by the method of Bergmann (36), *l-tryptophane* from casein by the method of Cole (37), and *carbamyl-l-leucine* from *l*-leucine by the action of KCNO (38).

All the other substances studied were of commercial origin. When necessary (on account, for instance, of contamination with ammonia), they were purified by adequate recrystallization.

Important amino acids omitted from present consideration are tyrosine, cystine, and glutamic acid. The first two could not, on account of their low solubility, be applied in sufficient concentration to give measurable effects. The other gave anomalous and irregular results, for which we cannot at present account.

Method

As a practical method of measuring the effect of an amino acid upon arginase activity we determined the apparent (or effective) concentration of an arginase solution acting on arginine in the presence of the amino acid, and compared this with the actual (or total) concentration as determined in action upon arginine alone. The arginase solution was always fully activated by cobalt, centrifuged, and adjusted to a concentration of between 10 and 15 units per ml. The methods used in preparing this solution and in measuring arginase activity have been described in a recent paper (39);

but in order to make the procedure perfectly clear we give here the details of a typical experiment with ornithine as inhibitor.

Into each of five Van Slyke-Cullen urea tubes were measured 2 ml. of a 1.875 per cent solution of arginine hydrochloride in phosphate-phenolsulfonate buffer solution, pH 8.4. To the first three were added respectively 5.0, 2.5, and 1.25 ml. of a 0.600 per cent ornithine monohydrochloride solution, followed by 0.0, 2.5, and 3.75 ml. of water. To the fourth were added 5.0 and to the fifth (serving as a blank control) 6.0 ml. of water. The first four tubes were set in a water bath at 37° along with a test-tube containing some of the prepared arginase solution. When all had reached the temperature of the bath, 1 ml. of the arginase was added seriatim to each of the tubes, and allowed to act for exactly 30 minutes. The action having then been terminated by acidification and boiling, each tube (together with the control) was neutralized and treated with 1 ml. of urease solution. The determination of urea produced was then completed in the usual way.

The results of this experiment are shown in Table I. In Table I the amount of the inhibitor, ornithine, is expressed on the one hand in terms of molar concentration (Column 3), on the other (Column 4) as the number of molecules, symbolized as n, per molecule of arginine. In Column 6 the urea values found are translated, by reference to the standard curve (see Fig. 1) of Hunter and Downs (39), into arginase concentrations, actual or apparent. Taking these to represent relative arginase activities, we then, in Column 7, express the activity manifested in the presence of each concentration of inhibitor as a fraction of the activity found (Tube 4) in its absence. Fractions so obtained and so defined we have designated "fractional activities," here symbolized as α. The calculation of fractional activities enables one to compare on a single basis the results of experiments with different absolute quantities of arginase. "Fractional inhibition" may be similarly calculated as $1 - \alpha$.

In the above experiment, conducted under the conditions which we have prescribed (39) for the routine determination of arginase, the final concentration of arginine was 0.0223 M. In the course of our inquiry occasion arose to study the inhibiting effect of some amino acids at other, higher arginine concentrations, those chosen being 0.0297, 0.0445, 0.0667, and 0.089 M. With these we used the same general method, modifying the details in the following two respects. (1) The concentration of the arginine-in-buffer solution was raised from 1.875 per cent to 2.5, 3.75, 5.625, and 7.5 per cent respectively. (2) For each new concentration we constructed its own individual reference curve. These special curves were prepared in exactly the same way as the original standard curve (39), only the arginine concentration being altered. It hardly seems necessary to present all four of them; but that for 0.089 M arginine is reproduced in Fig. 1, where it is properly adjusted to the scale of standard units. With each of the others, since relative values only were required, we were content to use an arbitrary or accidental scale. Table II shows the results of one experiment (again with ornithine) at each of the higher arginine concentrations.

TABLE I

Inhibitory Action of Ornithine

Arginine hydrochloride, 1.875 per cent in buffer solution, 2 ml.; ornithine hydrochloride, 0.6 per cent in water; arginase solution, 1 ml; total volume of each mixture, 8 ml.; final concentration of arginine, 0.0223 M

Tube No.	Ornithine HCl		$n = \frac{\text{ornithine}}{\text{arginine}}$	Urea N found	Arginase corresponding	Fractional activity $= \alpha$	Fractional inhibition $= 1 - \alpha$
	Volume of solution added	Final concentration					
	ml.	*M*		*mg*	*units per ml*		
1	5.0	0.0223	1.0	1.356	4.0	0.34	0.66
2	2.5	0.0111	0.5	1.832	6.0	0.51	0.49
3	1.25	0.00556	0.25	2.210	8.1	0.69	0.31
4	0.0	0.0	0.0	2.742	11.7	1.00	0.0

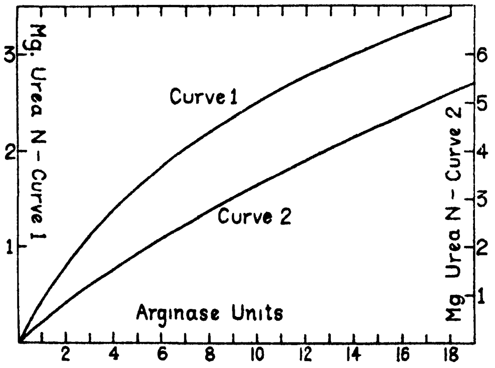


FIG. 1. Reference curves for measurement of arginase activity. Curve 1 (urea scale, left) is the standard curve for an arginine concentration of 0.0223 M. Curve 2 (urea scale, right) is a special curve for 0.089 M arginine.

Our method of assessing fractional activities is convenient in operation, and its results are reproducible; but it must be allowed that it is open to at least one objection. Fractional activity, in the meaning here assigned to that term, would be most correctly defined and measured as $\alpha = v/v_0$, where v_0 is the initial velocity of the reaction in the absence of the inhibitor and v the reduced velocity in its presence. With the indirect method we have employed α will be identical with v/v_0 only if the presence of the inhibitor does not affect the form of the time-action curve. This is not strictly the case. Our reported values of α, together with all calculated values derived from them, are accordingly subject to revision. Revision in the sense indicated could hardly affect the general picture presented, or the main conclusions suggested, by our results; but in the application to these of the concepts of enzyme kinetics it may at some points be necessary to exercise a certain reserve.

TABLE II

Inhibition by Ornithine at Various Arginine Concentrations

Each mixture contained, in a total volume of 8 ml., 2 ml. of arginine buffer solution and 1 ml. of arginase.

Concentration of arginine HCl		Ornithine HCl			n	Urea N found	Arginase corresponding	α
Initial	Final	Initial concentration	Volume added	Final concentration				
per cent	*M*	*per cent*	*ml*	*M*		*mg*	*units per ml*	
2.5	0.0297	0.800	5.0	0.0297	1.0	1.73	5.2	0.33
			2.5	0.0149	0.5	2.336	7.9	0.50
			0.0			3.515	15.7*	1.00
3.75	0.0445	1.200	5.0	0.0445	1.0	1.65	4.4	0.30
			2.5	0.0223	0.5	2.327	6.8	0.46
			1.25	0.0111	0.25	2.91	9.4	0.63
			0.0			3.877	14.8*	1.00
5.625	0.0667	1.800	5.0	0.0667	1.0	1.659	5.1	0.29
			2.5	0.0334	0.5	2.432	8.1	0.46
			0.0			4.401	17.6*	1.00
7.5	0.089	2.400	5.0	0.089	1.0	1.475	3.9	0.29
			2.5	0.0445	0.5	2.142	6.0	0.45
		1.200	5.0	0.0223	0.25	2.747	8.0	0.60
			2.5	0.0111	0.125	3.268	10.0	0.75
			0.0			4.106	13.3†	1.00

* Arbitrary units, differing for each arginine concentration.

† Standard units.

Theoretical

In describing the method, we have recorded a few of our experimental results. Before presenting the others, it will be convenient to review certain theoretical considerations which bear upon their interpretation. In this review we shall ignore the difficulty just mentioned, and shall use α as synonymous with v/v_0. S and I will be employed to designate molar concentrations of substrate and inhibitor respectively.

In the case of a *non-competitive* inhibitor, fractional activity (α) and fractional inhibition $(1 - \alpha)$ are independent of S, and, for any given physical environment, vary only with I. The exact relation between I and α will depend upon the mechanism of the inhibition. In the case in which 1 molecule of inhibitor inactivates 1 molecule of enzyme by combining with it reversibly at a different point from the substrate, the relation will be expressed (approximately) by the equation

$$1 - \alpha = C \tag{1}$$

in which C is a constant, characteristic of the particular inhibitor under consideration and identifiable as the dissociation constant of the enzyme-inhibitor complex. Equation 1 is the equivalent of Straus and Goldstein's (40) Equation 2A, and, like it, may be derived from Equation 2 of Easson and Stedman (41) under the simplifying assumption (usually justified) that only a negligible fraction of the total inhibitor present is actually combined with the enzyme. So long as this equation holds, S and I alike may be varied without affecting C. A graph of C (ordinates) against S (abscissae) will therefore be a horizontal straight line.

For the case of a purely *competitive* inhibitor, let K_s be the dissociation constant of the enzyme-substrate, and K_i that of the enzyme-inhibitor complex. Then, according to Michaelis and Menten (42),

$$K_i(S + K_s)\left(\frac{v_0}{v} - 1\right) = I \cdot K_s$$

Putting $1/\alpha$ for v_0/v and rearranging, we get

$$1 - \alpha = K_i + \frac{K_i}{K_s} \cdot S \tag{2}$$

If, now, the right-hand expression is symbolized by C, we get again Equation 1, $I \cdot \alpha/(1 - \alpha) = C$. Both forms of inhibition, therefore, show the same formal relation between α, C, and inhibitor concentration. The difference between them lies in the significance and behavior of C. With the non-competitive inhibitor of the type under consideration, C is a true constant, independent of both variables, I and S. With the competitive inhibitor,

$$C = K_i + \frac{K_i}{K_s} \cdot S \tag{3}$$

and its magnitude depends therefore not only upon two constants, K_s and K_i, but also upon a variable, S. Only for a given substrate concentration will $I \cdot \alpha/(1 - \alpha)$, *i.e.* C, remain constant. When S is varied, C will vary in the same direction. The graph of their relationship, as Equation

3 makes evident, will be a straight line, not horizontal as with a non-competitive inhibitor, but sloping upward from the axis of S.

With both types of inhibition it is evident, from Equation 1, that C is identical numerically with that concentration of inhibitor by which the activity of the enzyme is reduced to one-half. The effectiveness of an inhibitor, whether competitive or not, is therefore inversely proportional to its C value, or directly proportional to $1/C$. Of course, when competitive inhibition is concerned, effectiveness, thus judged, will vary with S.

Equation 1 can be transformed into

$$\alpha = \frac{C}{C + I} \tag{4}$$

which enables one, in any case where C is known, to calculate fractional activity at any concentration of inhibitor.

In the case of a competitive inhibitor, the experimental determination of C (from observed values of α and I) at two different substrate concentrations should enable one to estimate at once both K_s and K_i. For, if C_1 and C_2 are the values found for C at the substrate concentrations S_1 and S_2 respectively, then (by Equation 3) $C_1 = K_i + (K_i/K_s) \cdot S_1$ and $C_2 = K_i + (K_i/K_s) \cdot S_2$. Solving these simultaneous equations for K_s and K_i, we get

$$K_s = \frac{C_1S_2 - C_2S_1}{C_2 - C_1} \tag{5}$$

$$K_i = \frac{C_1S_2 - C_2S_1}{S_2 - S_1} \tag{6}$$

Theoretically, then, K_s and K_i can be obtained by making just two measurements of α, and hence of C, one at each of two different substrate concentrations and in the presence of any convenient concentration of inhibitor. From these measurements the dissociation constants would be calculated by Equations 5 and 6. In practice singly determined values of C will usually be subject to considerable error; and to obtain reasonably accurate results it may be necessary to make numerous observations at various concentrations both of substrate and of inhibitor. Under these circumstances the algebraic method of computing the constants is less convenient than a graphical one, of the kind employed by Lineweaver and Burk (43). Found values of C are plotted as ordinates against the known S values as abscissae. The straight line best fitting the experimental points will then represent Equation 3. Its intercept on the C axis will therefore be K_i, while its slope will be K_i/K_s. Intercept divided by slope will give K_s. Alternatively $-K_s$ may be found as the intercept of the line on the S axis; for when C is zero, Equation 3 becomes $S = -K_s$.

Results

In the presentation and discussion of our results we employ the symbols A, O, and LY to represent respectively molar concentrations of arginine, ornithine, and lysine. Experimental values of α are used to calculate C by Equation 1. The C values specific to ornithine and lysine are symbolized as C_o and C_{ly}, while K_a, K_o, and K_{ly} designate the dissociation or Michaelis constants of the compounds formed by arginase with arginine, ornithine, and lysine respectively.

Inhibition by Ornithine—With this amino acid we made experiments at all five of the different concentrations of arginine already specified, while the concentration of the ornithine itself was varied from 0.178 M to 0.00139 M. At the lowest arginine level (0.0223 M) the molecular ratio of ornithine to arginine ranged from 8 to 1/16, at the highest (0.089 M) from 1 to 1/16. In Tables I and II we have already given complete protocols of several of these experiments. In Table III we present in summary form the results of all.

Inspection of these results shows that at any one concentration of arginine ($A = 0.0223$ excepted) they are in satisfactory conformity with Equation 1, α varying with the ornithine concentration in such a manner that C_o remains substantially constant. In the exceptional instance C_o increases significantly as the ornithine concentration falls. Examples of a similar trend appear also among the results for lysine, leucine, etc. (Tables IV and V). Concerning its possible import we have no unambiguous evidence. For the present, therefore, we propose to ignore it, and to treat C_o at $A = 0.0223$ provisionally as a constant. Its most probable value we have taken as the mean (0.0121) of all values found at ornithine concentrations between 0.089 and 0.00278 M.

When the arginine concentration is varied, C_o no longer remains constant, but varies in the same direction. The relation between the two variables is shown graphically in Fig. 2. Here we have entered as separate points only the mean value of C_o at each concentration of arginine, but for each such mean have indicated the number of individual values included and the range within which they varied. All of the points lie near a straight line inclined at a considerable angle to the axis of A. This behavior, it has been shown, is that to be expected of a competitive inhibitor. The line drawn was fitted to the collective data by the method of least squares, and is represented by the equation $C_o = 0.00411 + 0.355A$. This gives to K_o the value 0.0041, while K_a is $0.0041/0.355 = 0.0116$ (with 95 per cent fiducial limits 0.0101 to 0.0131).

Inhibition by Lysine—The experiments with lysine were not so numerous as those with ornithine, but were conducted on the same plan. The results are summarized in Table IV. They indicate for lysine an inhibitory effect

TABLE III

Inhibition of Ornithine at Various Concentrations of Arginine

A = molar concentration of arginine; O = molar concentration of ornithine. The number of observations made at each value of O is given in parentheses.

A	O	α		C_0	
		Range	Mean	Mean for given O	Grand mean for given A
0.0223	0.178 (2)		0.056*	(0.0102)	0.0121
	0.089 (2)		0.11*	0.0104	
	0.0445 (7)	0.19–0.21	0.200	0.0111	
	0.0223 (10)	0.33–0.36	0.344	0.0117	
	0.0111 (14)	0.51–0.55	0.528	0.0124	
	0.00556 (7)	0.69–0.71	0.700	0.0130	
	0.00278 (2)		0.83*	0.0136	
	0.00139 (2)	0.90–0.91	0.905	(0.0132)	
0.0297	0.0297 (3)	0.32–0.33	0.327	0.0145	0.0149
	0.0148 (4)	0.50–0.51	0.503	0.0150	
	0.0074 (1)		0.68	0.0157	
0.0445	0.0445 (2)		0.30*	0.0191	0.0190
	0.0223 (4)	0.45–0.46	0.458	0.0188	
	0.0111 (2)	0.63–0.64	0.635	0.0193	
0.0667	0.0667 (1)		0.29	0.0272	0.0277
	0.0334 (1)		0.46	0.0284	
	0.0167 (3)	0.62–0.63	0.622	0.0275	
	0.00834 (2)		0.77*	0.0279	
0.089	0.089 (4)	0.29–0.30	0.292	0.0367	0.0358
	0.0445 (3)	0.45–0.46	0.453	0.0368	
	0.0223 (5)	0.60–0.63	0.612	0.0352	
	0.0111 (7)	0.75–0.77	0.760	0.0352	
	0.00556 (2)	0.86–0.87	0.865	0.0356	

* Individual values of α identical

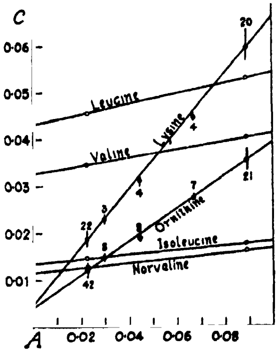


FIG. 2. Relation of C to arginine concentration (A) for the l forms of six amino acids. Dots or circles represent mean values of C. For ornithine and lysine the number of observed values included in each mean is given, and their range is indicated by a vertical line.

at least 0.6 times as great as that of ornithine, 0.6 being the minimum value of the ratio $C_o:C_{ly}$ at any given A. With this quantitative difference the action of lysine conforms in practically every respect to the pattern set by ornithine. In particular the C_{ly} values, plotted against A, lie on, or sufficiently near to, a straight line with a considerable upward slope. Lysine is therefore a competitive inhibitor. The line fitted to the data by least squares, and drawn through the lysine points in Fig. 2, has the equation $C_{ly} = 0.00476 + 0.616A$. From this equation it would appear that $K_{ly} = 0.0048$ while $K_a = 0.0077$ (with 95 per cent fiducial limits 0.00616 to 0.00836). The difference between this value for K_a and that indicated by the ornithine experiments will be commented upon in the discussion.

TABLE IV

Inhibition by Lysine at Various Concentrations of Arginine

A = molar concentration of arginine; LY = molar concentration of lysine. The number of observations at each value of LY is given in parentheses.

A	LY	α		C_{ly}	
		Range	Mean	Mean for given LY	Grand mean for given A
0.0223	0.178 (1)		0.084	(0.0163)	0.0187
	0.089 (1)		0.16	0.0170	
	0.0445 (5)	0.28–0.30	0.290	0.0182	
	0.0223 (7)	0.45–0.47	0.454	0.0185	
	0.0111 (4)	0.63–0.65	0.638	0.0196	
	0.00556 (3)		0.78*	0.0197	
	0.00278 (2)		0.87*	0.0186	
	0.00139 (2)		0.93*	(0.0185)	
0.0297	0.0594 (1)		0.28	0.023	0.0230
	0.0297 (1)		0.43	0.022	
	0.0148 (1)		0.62	0.024	
0.0445	0.089 (1)		0.26	0.031	0.0312
	0.0445 (1)		0.40	0.030	
	0.0223 (1)		0.59	0.032	
	0.0111 (1)		0.74	0.032	
0.0667	0.134 (1)		0.25	0.045	0.0450
	0.0667 (1)		0.40	0.044	
	0.0334 (1)		0.58	0.046	
	0.0167 (1)		0.73	0.045	
0.089	0.089 (6)	0.39–0.40	0.393	0.0576	0.0597
	0.0445 (6)	0.56–0.59	0.572	0.0594	
	0.0223 (8)	0.73–0.74	0.734	0.0615	

* Individual values of α identical.

Inhibition by Leucine, Isoleucine, Valine, Norvaline, and α-Aminobutyric Acid—With amino acids other than ornithine and lysine we carried out experiments at not more than two concentrations of arginine (0.0223 and 0.089 M), in some cases only at one (0.0223 M). In Table V we present first the results obtained with two structurally isomeric leucines, two valines, and α-aminobutyric acid. All five, it will be seen, are highly active inhibitors, exerting effects which in some cases and under some conditions are not inferior to those of ornithine or lysine. All, however, differ from the diamino acids in this respect, that to a 4-fold increase of the substrate concentration they respond with a relatively small increase of C. For the leucines and valines the contrast of behavior thus expressed is exhibited graphically in Fig. 2,[1] the compass of which is too small to accommodate the

TABLE V

Inhibition by Leucine, Isoleucine, Valine, Norvaline, and α-Aminobutyric Acid

A = molar concentration of arginine; I = molar concentration of inhibiting amino acid.

Inhibitor	I	A = 0.0223					A = 0 089				
		No of observations	α		C		No. of observations	α		C	
			Range	Mean	Mean for given I	Grand mean for given A		Range	Mean	Mean for given I	Grand mean for given A
l(−)-Leucine	0.089	3	0.30–0.32	0.307	0.0394	0.0456	2		0.36	0.0501	0.0532
	0.0445	6	0.49–0.51	0.502	0.0449		4	0.54–0.56	0.55	0.0544	
	0.0223	7	0.67–0.71	0.687	0.0490		1		0.71	0.0546	
dl-Leucine	0.089	3	0 53–0.55	0.540	0 105	0.108					
	0.0445	3	0.70–0.72	0.713	0.111						
	0.0223	3	0.82–0.84	0.830	0.109						
dl-Isoleucine	0 089	2	0.22–0.24	0.230	0.0266	0.0296	2	0.26–0.28	0.270	0.0329	0.0359
	0.0445	4	0.38–0.43	0.397	0.0293		4	0.43–0.45	0.440	0.0350	
	0.0178	2	0.63–0.67	0.650	0.0331		2	0.68–0.71	0.695	0.0406	
l(+)-Isoleucine	0.089	1		0.126	0.0128	0.0136					
	0.0445	1		0.234	0.0136						
	0.0223	1		0.392	0 0144						
dl-Valine	0.178	5	0.26–0.29	0.274	0.0671	0.0694	5	0.30–0.33	0.310	0.0800	0.0812
	0.089	11	0.42–0.48	0.440	0.0700		5	0.46–0.49	0.476	0.0808	
	0.0445	3	0.60–0.64	0.613	0 0705		5	0.64–0.67	0.653	0.0838	
dl-Norvaline	0.089	3	0.20–0.22	0.210	0 0236	0.0255	3	0.25–0.27	0.263	0.0317	0.0330
	0.0445	3	0.34–0.39	0.360	0.0250		3	0.41–0.43	0.423	0.0326	
	0.0178	3	0.59–0.64	0.610	0 0278		3	0.64–0.68	0.660	0.0346	
dl-α-Aminobutyric acid	0.356	2	0.33–0.35	0.340	0 183	0.168	2		0.39	0.228	0.206
	0.178	4	0.48–0.50	0.490	0.171		2		0.53	0.201	
	0.089	3	0.62–0.64	0.633	0.154		2		0.68	0.189	

---

[1] In plotting this figure we have estimated the C values of *l*-valine, *l*-norvaline, and *l*-isoleucine as one-half of the mean values found for the corresponding *dl* forms. Justification for this procedure will be given later.

data for aminobutyric acid. The figure makes it clear that inhibition by the amino acids of Table V is of a quite different character from that exerted by ornithine and lysine. It must then be mainly, if not wholly, non-competitive.

If it were purely non-competitive and of the type represented by Equation 1, C would in theory remain the same under all variations of inhibitor or arginine concentration, and the relevant lines in Fig. 2 would be exactly horizontal. In congruence with the constancy of C, α would be independent of A, and would vary only with the concentration of inhibitor. The data are in approximate conformity with these criteria; yet they present inconsistencies, which are not wholly negligible. Thus not only is C in each case significantly higher at $A = 0.089$ than at $A = 0.0223$, but (the case of aminobutyric acid excepted) even at constant A it rises as the inhibitor concentration falls. These discrepancies, it can be shown, are just of the kind to be expected when the action of an inhibitor, although predominantly non-competitive, is in part also competitive. Such a dual action on the part of the leucines and valines cannot, however, without further evidence be regarded as more than a possibility. With aminobutyric acid the behavior of C at constant A is inconsistent with the intervention of a competitive factor.

A point well illustrated by Fig. 2 is that, when inhibitors of different types are compared, their relative efficiencies depend upon the substrate concentration. Thus at $A = 0.02$ the most potent inhibitor of arginase is the competitive ornithine, the least potent the non-competitive leucine; but at $A = 0.09$ ornithine is only half as effective as norvaline and isoleucine, while even leucine now surpasses the competitive lysine.

Relation of Inhibitory Action to Configuration—Many of the amino acids having been used in their racemic forms only, it became important to know whether these exerted the same effect as their naturally occurring optically active isomers. As bearing upon this question we report the following observations.

A specially pure sample of *d*(+)-leucine, of which we had only enough for one experiment, was applied in 0.0445 M concentration and in the presence of 0.0223 M arginine. The urea N found was 3.105 mg. without, and 3.06 mg. with the leucine. The difference is within the experimental error, so that for the case of *d*(+)-leucine inhibitory action upon arginase is shown to be absent or negligible. This conclusion can probably be extended to all amino acids of the *d* series.

If the *d*-amino acids are inactive, it is to be expected that a racemic acid will have just half the effect of its *l* component. This expectation was realized quite exactly in a comparison of *l*(+)- and *dl*-isoleucine. The data, to be found in Table V, show that 0.0445 and 0.0223 M *l*(+)-isoleucine give the same α values as twice the respective concentrations of *dl*-isoleucine. The corresponding C values for the first are accordingly just half those for the second. If the mean C values depart somewhat from an exact 1:2 ratio, it is only because the two ranges of α overlap, so that the drift of C with isoleucine concentration affects the two means in opposite directions.

When *l*- and *dl*-leucine are similarly compared, the result is less nearly ideal, for with either a 0.0223 or a 0.0445 M concentration of the first α is smaller than with a double concentration of the second. At each pair of levels the ratio of C values is about 1:2.3. The divergence from the theoretical ratio is, to be sure, not very great; and, in any case, its direction is such as to negative any inhibitory action on the part of *d*-leucine.

From these observations we conclude that the only amino acids having any appreciable inhibitory effect upon arginase are those of the *l* series, that a racemic amino acid exerts half (or nearly half) the effect of its *l* component, and that, accordingly, the C value of any *l*-amino acid may without serious error be estimated as half the observed C of its racemic form. In such an estimate the effect of the "natural" isomer will at least not be exaggerated.

Inhibition by Other α-Amino Acids and by Some Amino Acid Derivatives—Amino acids (or amino acid derivatives) other than those already considered were tested less thoroughly. Table VI gives a condensed statement of the results obtained at an arginine concentration of 0.0223 M. This statement shows only, for each substance named, the range of concentrations employed and the C values (range and mean) yielded by the primary data. The corresponding values of α, here omitted, may, if desired, be calculated by means of Equation 4. It will be seen that among the substances listed one only, α-carbamylornithine, has no inhibitory effect whatsoever. On the other hand with none, save cysteine, is the effect of more than moderate magnitude, and with some (glycine and histidine) it is very slight. It may be added that with norleucine, phenylalanine, proline, and citrulline parallel experiments at 0.089 M arginine indicated a non-competitive type of inhibition.

That cysteine, which has been the subject of many earlier investigations (6–20), should have a strongly inhibitory action was not fully anticipated; for at alkaline reactions and especially in an aerobic environment (conditions which prevailed in our experiment) it has most often been found to be an activator of arginase. It would appear, however, that activation is a complex phenomenon, requiring the concurrence of certain substances commonly present in crude preparations of arginase. With purified arginase cysteine is inhibitory at all reactions, even at a pH of 9.5 (7, 13, 14, 16). Our arginase was at least relatively pure, so that the observed effect at pH 8.4 is not irreconcilable with previous experience.

Miscellaneous Observations—Since arginase is strongly inhibited by ornithine, it is of interest to know how far, if at all, it is affected by the other product of its action, urea. Most earlier workers have reported either no effect (1, 4) or only a slight inhibition (2); but Vovtchenko (3) records marked inhibition, apparently by 0.55 per cent (0.092 M) urea. Our own experience is that in concentrations up to 0.089 M urea has no influence at all upon the action of arginase.

TABLE VI

Inhibition by Various Amino Acids or Amino Acid Derivatives

The arginine concentration was in all cases 0.0223 M. *n* signifies the ratio of inhibitor to arginine concentration. Inhibitor concentrations are to be calculated therefore as $I = 0.0223n$. The figures in parentheses in the *n* column indicate the number of separate observations.

	n (range)	*C*	
		Range	Mean
Glycine	40–20 (4)	5.5 –4.5	5.0
l(+)-Alanine	10– 2.5 (3)	0.38 –0.37	0.37
dl-Norleucine	2– 1 (6)	0.45 –0.53	0.49
l(+)-Aspartic acid*	8– 2 (5)	0.33 –0.35	0.34
dl-Serine	10– 5 (2)	1.37 –1.28	1.33
dl-Phenylalanine	4– 1 (8)	0.26 –0.38	0.35
l(–)-Tryptophane	2– 1 (2)	0.70 –0.72	0.71
l(–)-Cysteine	4– 1 (3)	0.035–0.050	0.043
dl-Methionine	4– 2 (2)	0.90 –0.85	0.88
l(–)-Histidine	8– 4 (5)	4.0 –3.5	3.8
l(–)-Proline	10– 2.5 (3)	0.30 –0.27	0.28
l(–)-Hydroxyproline	10– 2.5 (3)	0.67 –0.37	0.52
l(+)-Citrulline	16– 4 (3)	0.40 –0.55	0.47
l(+)-α-Carbamylarginine	2– 0.5 (6)	0.27 –0.31	0.29†
l(–)-Argininic acid	8– 2 (3)	1.0 –1.7	1.4†
l(+)-α-Carbamylornithine	8– 1 (4)		∞
Formyl-*l*(+)-valine*	10– 2.5 (6)	0.63 –0.45	0.55
Carbamyl-*l*-leucine*	8– 1 (5)	0.44 –0.54	0.49

* Used in solutions neutralized with NaOH.

† These are corrected values, in which an allowance has been made for urea produced by arginase from the inhibitor itself (35, 44–47). The corrections, based as they are upon parallel controls containing no arginine, are probably too large, and the values of *C* therefore too small. The true values are probably at least twice as great.

Other substances found to be without effect were *dl*-β-alanine, tested in concentrations up to 0.223 M, *dl*-β-amino-*n*-butyric acid (0.356 M), *dl*-δ-amino-*n*-valeric acid (0.445 M), and *dl*-ε-amino-*n*-caproic acid (0.445 M). From these instances it would appear that the α-amino acids are the only ones which interfere with the action of arginase.

A few experiments were made with proteins, the maximum concentration employed being in each case 2.5 per cent. Casein and gelatin gave definitely negative results. Serum albumin showed only a slight depressant action, possibly not significant. On the other hand 2.5 per cent of Witte's peptone caused a 15 per cent reduction of arginase activity. These results are only in partial agreement with those of Klein and Ziese (48), who obtained not only a notable inhibiting effect with peptone, but also definite, although smaller, effects with albumin and globulin. A possible source of the discrepancy is the use by Klein and Ziese of a very crude preparation of arginase. The effect of peptone is probably to be attributed to relatively simple constituents of polypeptide character or even to free amino acids.

DISCUSSION

Although both ornithine and lysine were shown to be competitive inhibitors, experiments with the first yielded for K_a a value (0.0116) 50 per cent higher than that (0.0077) found with the second. Since the 95 per cent confidence ranges of the two estimates do not overlap, the difference may be taken to be statistically significant. The meaning to be attached to it is uncertain; but one feasible explanation would be that ornithine (as yielding the higher estimate of K_a) not only competes with arginine for the active group of arginase, but also inhibits that enzyme in a non-competitive manner. Such a 2-fold action on the part of ornithine would not be at all surprising; for the corresponding monoamino acid, norvaline, is an inhibitor of the same order of potency as ornithine itself, but in its action non-competitive. On the other hand norleucine, corresponding to lysine, is only feebly inhibitive (see Tables VI and VII). It might therefore actually be expected that ornithine would exert both types of inhibition, but that lysine would have little, if any, non-competitive action. On the basis of these expectations the true value of K_a would be that found in the lysine experiments, namely 0.0077.

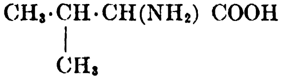
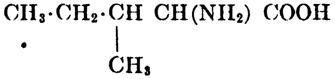
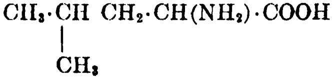
Unfortunately the data for ornithine furnish in themselves no conclusive evidence either for or against the hypothesis of a mixed effect. The exact value of K_a remains therefore in doubt. Any error in the estimation of K_a involves a corresponding error of the same sign in that of K_o or K_{ly}. In these circumstances we are unable on the basis of the present experiments to assign a precise value to any one of the three dissociation constants. All that can be safely deduced from the data is that K_a is of the order 0.01, that K_o is probably not greater than 0.004, and that K_{ly} is unlikely to be less than 0.0048. These values, however indefinite, leave no doubt that the affinity of arginase is greater (although not much greater) for ornithine than for lysine, and greater for either of these inhibitors than for its substrate.

The notable affinity of lysine for arginase would lead one to expect that the corresponding arginine analogue (ε-guanido-α-amino-*n*-caproic acid or homoarginine) would likewise possess affinity, and that it would accordingly be susceptible to the action of the enzyme. This expectation is realized (33, 49), although the rate at which homoarginine is hydrolyzed is relatively small.

The α-monoamino acids, most of which have been shown, and all of which may be presumed, to be (exclusively or predominantly) non-competitive inhibitors, show a very wide range of effectiveness. It becomes of interest to inquire, therefore, what factors of constitution determine their relative activities. Among the most likely factors are length and branching of the carbon chain; and data elucidative of their influence are accordingly collected in Table VII. It there appears that in the homologous series of straight chain monoaminomonocarboxylic acids inhibitory activity increases regularly and rapidly from glycine with 2 carbon atoms to norvaline with 5, but suffers a notable diminution, when the chain is extended, in norleucine, to 6 carbon atoms. For some reason, then, the 5-carbon straight chain represents a critical length, at which non-competitive interference with the action of arginase becomes maximal. The effect of branching appears at first sight to be quite irregular; for in the valines it is associated with a diminished, in the leucines with an increased, inhibitory activity. The facts may, however, be systematized in the following way.

TABLE VII

Effect of Length and Branching of Carbon Chain

Amino acid	Structure	C for l form* with $A = 0.0223$	$\frac{1}{C}$
Glycine	$CH_2(NH_2)\cdot COOH$	5.0	0.2
Alanine	$CH_3\ CH(NH_2)\ COOH$	0.37	2.7
α-Aminobutyric acid.	$CH_3\cdot CH_2\cdot CH(NH_2)\cdot COOH$	0.084	11.9
Norvaline	$CH_3\cdot CH_2\ CH_2\cdot CH(NH_2)\cdot COOH$	0.0128	78.1
Norleucine	$CH_3\cdot CH_2\cdot CH_2\cdot CH_2\cdot CH(NH_2)\cdot COOH$	0.25	4.0
Valine	 $CH_3\cdot\underset{\displaystyle CH_3}{\underset{\vert}{CH}}\cdot CH(NH_2)\ COOH$	0.0347	28.8
Isoleucine	 $CH_3\cdot CH_2\cdot\underset{\displaystyle CH_3}{\underset{\vert}{CH}}\ CH(NH_2)\ COOH$	0.0148	67.6
Leucine	 $CH_3\cdot\underset{\displaystyle CH_3}{\underset{\vert}{CH}}\ CH_2\cdot CH(NH_2)\cdot COOH$	0.0458	21.9

* C values found for *dl*-amino acids are here halved

An added methyl group has the same *kind* of effect, whether it is introduced as a branch or at the end of a chain. Thus both 5-carbon amino acids are more actively inhibitory than α-aminobutyric acid, and all three 6-carbon acids less active than norvaline. In either case the *magnitude* of the specific effect increases with the distance between the extra $—CH_3$ and the $—NH_2$ group. Norvaline, accordingly, inhibits more strongly than valine, while norleucine is less active than leucine, and this again less active than isoleucine. In the last, where $—CH_3$ and $—NH_2$ are on adjacent carbons, the effect is so small that inhibitory activity falls little below the norvaline level.

Substituent groups other than $—CH_3$ have diverse effects upon inhibitory activity. In Table VIII *l*(+)-alanine and various β derivatives thereof are arranged in the order of increasing potency as inhibitors. It will be seen that the effect of alanine is reduced to one-tenth by imidazole substitution, halved by indole or —OH, hardly affected by —COOH, doubled by $—C_6H_5$, doubled again by $—CH_3$, and doubled once more by —SH. The weakening influence of —OH is confirmed by a comparison of proline ($C = 0.28$) and hydroxyproline ($C = 0.52$).

TABLE VIII

Effect of Substitutions in Methyl Group of Alanine

Amino acid	Substituting radical	C for *l* form* with $A = 0.0223$	$\frac{1}{C}$
Histidine	Imidazole	3.8	0.26
Tryptophane	Indole	0.71	1.4
Serine	—OH	0.67	1.5
Alanine	—H	0.37	2.7
Aspartic acid	—COOH	0.34	2.9
Phenylalanine	$—C_6H_5$	0.175	5.7
α-Aminobutyric acid	$—CH_3$	0.084	11.9
Cysteine	—SH	0.043	23.2

* C values found for *dl*-amino acids are here halved.

Substitution in the amino group always reduces inhibitory activity. Thus the carbamyl derivative of leucine ($C = 0.49$) is only about one-tenth, the formyl derivative of valine ($C = 0.55$) only about one-twelfth, as active as its parent amino acid. The persistence of some residual activity in these instances shows that non-competitive inhibition is not wholly dependent upon an intact α-amino group. With regard to competitive inhibition, where two amino groups come into play, the situation is not in all respects so clear. The complete inertness of α-carbamylornithine, considered by itself, would suggest that an unsubstituted α-amino group is here indispensable; but against this must be set the fact that α-carbamylarginine (and even argininic acid) exerts a measurable effect of inhibition, which, since it is associated with susceptibility to the enzyme, is presumably of competitive type. Less ambiguous are the effects of substitution in a terminal $—NH_2$. Here the entry of the basic guanyl radical, as in arginine or homoarginine, reduces affinity for the active center of the enzyme; but the acyl group carbamyl abolishes it completely. Citrulline, in contrast with ornithine, is a non-competitive inhibitor, behaving in fact like a moderately active monoamino acid.

It should be noted, in conclusion, that all our experiments were carried out at a uniform pH of 8.4, and that at any other pH the various constants, which we have attempted to evaluate, might have sensibly different magnitudes. Data presented by Van Slyke (50) suggest that K_a at pH 9.2 is about 0.02. On the other hand Damodaran and Narayanan (51) found even at pH 9.4 a value (0.0118) identical with that yielded by our ornithine experiments.

We are greatly indebted to Professor D. B. DeLury of the Department of Mathematics, University of Toronto, Professor H. O. L. Fischer of the Department of Chemistry, and Dr. J. M. R. Beveridge of the Banting and Best Department of Medical Research. To the first we owe the statistical analysis of the data, to each of the others generous gifts of material.

SUMMARY

The action of arginase upon arginine at pH 8.4 is inhibited by all α-amino acids of the naturally occurring *l* configuration, but not by *d*-α-amino acids, amino acids having the amino group in other than the α position, urea, or native proteins.

At any one concentration of arginine the influence of varying concentrations of inhibiting amino acid can in all cases be expressed by the equation $I \cdot \alpha/(1 - \alpha) = C$, in which I is the concentration of the inhibitor, α is the resultant "fractional activity," and C is, for each amino acid, constant (or approximately so) and characteristic.

With the monoamino acids C is (nearly) independent, not only of the inhibitor concentration but also of the concentration of arginine. With ornithine and lysine this is not the case. The difference is interpreted to mean that the monoamino acids are non-competitive inhibitors, the diamino acids competitive. In the first case C is probably to be taken as the dissociation constant of a reversible complex involving some point of the enzyme molecule other than its active center. In the second $C = K_i + (K_i/K_a) \cdot A$, where K_a is the dissociation constant of arginine-arginase, K_i that of inhibitor-arginase, and A the molar concentration of arginine. In some instances one type of inhibition is perhaps combined with a minor degree of the other.

With any purely competitive inhibitor the determination of C at two or more different concentrations of arginine enables one to estimate both K_a and K_i. The application of this method with ornithine and lysine indicated that K_a is about 0.01, while K_i for ornithine is not more than 0.004 and for lysine not less than 0.0048.

Of the two competitive inhibitors the more potent is ornithine. Among the non-competitive, the degree of inhibitory action exerted depends in part upon the length and shape of the carbon chain, in part upon the nature and position of any distinctive substituent radicals. Substitution in an amino group has in general the effect of reducing inhibitory activity or affinity for arginase.

BIBLIOGRAPHY

1. Gross, E., *Z. physiol. Chem.*, **112,** 236 (1921).
2. Edlbacher, S., and Zeller, A , *Z. physiol. Chem.*, **242,** 253 (1936).
3. Vovtchenko, G. D., *Arch. sc biol.*, U. S S. R., **42,** 89 (1936).
4. Kitagawa, M., *J. Agr. Chem Soc. Japan*, **15,** 267 (1939); cited from *Chem. Abstr.*, **33,** 2545 (1939).
5. Bach, S. J., and Williamson, S., *Nature*, **150,** 575 (1942).
6. Salaskin, S. S., and Solowjew, L., *Z. physiol. Chem.*, **200,** 259 (1931); **205,** 171 (1932).
7. Salaskin, S. S., and Solowjew, L., *Biochem. Z.*, **250,** 503 (1932).
8. Edlbacher, S., Kraus, J., and Walter, G., *Z. physiol. Chem.*, **206,** 65 (1932).
9. Klein, G., and Ziese, W., *Z. physiol. Chem.*, **211,** 23 (1932).
10. Klein, G , and Ziese, W , *Z. physiol. Chem.*, **213,** 201 (1932).
11. Waldschmidt-Leitz, E., *Z. physiol. Chem* , **214,** 75 (1932).
12. Edlbacher, S., Kraus, J., and Leuthardt, F , *Z. physiol. Chem.*, **217,** 89 (1933).
13. Klein, G , and Ziese, W., *Z physiol Chem* , **222,** 187 (1933).
14. Klein, G , and Ziese, W., *Z physiol. Chem.*, **229,** 209 (1934).
15. Karrer, P., and Zehender, F., *Helv chim. acta*, **17,** 737 (1934).
16. Leuthardt, F., and Koller, F., *Helv chim. acta*, **17,** 1030 (1934).
17. Purr, A , and Weil, L., *Biochem J* , **28,** 740 (1934).
18. Weil, L., *J. Biol. Chem.*, **110,** 201 (1935).
19. Hellerman, L , and Perkins, M. E., *J. Biol. Chem.*, **112,** 175 (1935–36).
20. Kitagawa, M , and Watanabe, T., *J. Agr Chem. Soc. Japan*, **14,** 779 (1938); cited from *Chem. Abstr.*, **32,** 9104 (1938).
21. Kitagawa, M , *J. Agr. Chem. Soc Japan*, **15,** 267 (1939); cited from *Chem. Abstr.*, **33,** 2545 (1939).
22. Hunter, A , *Biochem J.*, **33,** 27 (1939).
23. Rice, E. E., *J. Biol. Chem.*, **131,** 1 (1939).
24. Lawrow, D., *Z. physiol. Chem.*, **28,** 585 (1899).
25. Henderson, Y., *Z. physiol Chem.*, **29,** 320 (1900).
26. Szydlowski, L., *Monatsh. Chem.*, **27,** 821 (1906).
27. Kurtz, A C., *J. Biol Chem.*, **140,** 705 (1941).
28. Gilson, L. E., *J. Biol. Chem.*, **124,** 281 (1938).
29. Vickery, H. B., *J. Biol. Chem.*, **71,** 303 (1926–27).
30. Fischer, E., and Warburg, O., *Ber. chem. Ges.*, **38,** 3997 (1905).
31. Fischer, E., *Ber. chem. Ges.*, **39,** 2320 (1906).

32. Boon, W. R., and Robson, W., *Biochem. J.*, **29**, 2573 (1935).
33. Felix, K., and Schneider, H., *Z. physiol. Chem.*, **255**, 132 (1938).
34. Gornall, A. G., and Hunter, A., *Biochem. J.*, **33**, 170 (1939).
35. Hunter, A., *Biochem. J.*, **32**, 826 (1938).
36. Bergmann, M., *J. Biol. Chem.*, **110**, 471 (1935)
37. Cole, S. W., Practical physiological chemistry, Cambridge, 9th edition, 127 (1933).
38. Dakin, H. D., *Am. Chem. J.*, **44**, 48 (1910).
39. Hunter, A., and Downs, C. E., *J. Biol. Chem.*, **155**, 173 (1944).
40. Straus, O. H., and Goldstein, A., *J. Gen. Physiol.*, **26**, 559 (1943).
41. Easson, L. H., and Stedman, E., *Proc. Roy. Soc. London, Series B*, **127**, 142 (1936).
42. Michaelis, L., and Menten, M. L., *Biochem. Z.*, **49**, 333 (1913).
43. Lineweaver, H., and Burk, D , *J. Am. Chem. Soc.*, **56**, 658 (1934).
44. Calvery, H O., and Block, W. D., *J Biol. Chem.*, **107**, 155 (1934).
45. Felix, K., and Müller, H., *Z. physiol. Chem* , **240**, 1 (1936).
46. Hellerman, L., and Stock, C. C., *J. Biol Chem.*, **125**, 771 (1938).
47. Hunter, A., and Woodward, H. E., *Biochem. J.*, **35**, 1298 (1941).
48. Klein, G., and Ziese, W., *Z. physiol Chem* , **213**, 217 (1932).
49. Greenstein, J. P., Jenrett, W. V , Mider, G. B., and White, J., *J. Nat. Cancer Inst.*, **1**, 687 (1941)
50. Van Slyke, D. D., in Nord, F. F., and Werkman, C. H., Advances in enzymology and related subjects, New York, **2**, 33 (1942).
51 Damodaran, M , and Narayanan, K. G. A., *Biochem J.*, **34**, 1449 (1940).

THE NATURE OF SOME NEW DIETARY FACTORS REQUIRED BY GUINEA PIGS

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The recognition of three new dietary essentials required by guinea pigs was described from this laboratory in 1942 (1). These factors were shown to be necessary in addition to the vitamins recognized at that time, and, for lack of more precise terms, were designated GPF-1, GPF-2, and GPF-3. Shortly thereafter the existence of these three factors was indicated independently by Sober *et al.* (2), and the need of guinea pigs for dietary factors as yet unidentified was pointed out by Hogan and Hamilton (3). We wish now to report the identification of GPF-1 as folic acid, the substitution of casein (or arginine, cystine, and glycine) plus cellulose[1] for GPF-2, and the concentration of GPF-3 Some speculation concerning the relation of this last factor to the growth of other organisms will also be presented.

When attempts were made to isolate GPF-2, difficulty was experienced in obtaining the factor in solution. It was then observed that active preparations derived from various plant materials were always rich in protein and in fiber. When the protein level in the basal diet was increased from the original 18 to 30 per cent and fiber was added as cellulose, the need for GPF-2 was no longer apparent. The effect of the extra casein was duplicated by a mixture of arginine, cystine, and glycine. It has not yet been determined whether all three amino acids or only one or two were responsible. Similar situations with respect to protein and to the above amino acids have been observed in chicks (4) and in rats (5). In the presence of 30 per cent casein the omission of cellulose resulted in suboptimal rates of growth, but mortality on such a fiber-deficient diet was low. A requirement for cellulose has not been encountered previously in laboratory animals, although the desirability of roughage in the diet of certain species has long been known in animal husbandry. In spite of the fact that the cellulose (Cellu flour) used had been purified, it was not certain whether its activity was due to cellulose or to some unidentified impurity contained therein. However, cellophane, which is prepared by a series of operations which tend to eliminate adherent impurities, was active.

The substitution of purified casein and cellulose for preparations of GPF-2 was largely responsible for the identification of GPF-1 and the concentration of GPF-3, for it permitted the use of a basal diet in which all constituents were highly purified. This basal diet contained glucose, casein, cellulose, inorganic salts, and the known vitamins as used previously (1). On this diet guinea pigs grew poorly and died within 2 to 4 weeks. The addition of solubilized liver extract[2] (Wilson's fraction L), however, produced a ration adequate for good growth for at least 15 weeks. In view of this fact, fractionations of the liver extract were undertaken in order to identify the active materials. Separation of two dietary essentials, GPF-1 and GPF-3, was accomplished.

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[1] The cellulose used in this work was in the form of Cellu flour.

GPF-1 was identified as folic acid.[3] This was made possible only when it was found how to make GPF-3 preparations low in folic acid. In the presence of such GPF-3 concentrates, either folic acid concentrates or the crystalline material produced good growth. In the absence of the GPF-3 preparation folic acid was ineffective. The properties of GPF-1 previously described (1) were similar to those of folic acid, and since concentrates of GPF-1 were rich in folic acid as measured by the growth of *Lactobacillus casei*, it was concluded that GPF-1 and folic acid were identical.

Concentrates of GPF-3 have been made by procedures used in this laboratory (8) to purify the new growth factor, strepogenin, required by hemolytic streptococci, and by certain other bacteria (9). The presence of GPF-3 in such preparations may be coincidental, but it at least demonstrates certain similarities in properties of the bacterial growth factor and the new dietary essential. GPF-3 was not readily precipitated by lead acetate or adsorbed by norit, and, although it was insoluble in alcohol, it dissolved in alcoholic HCl. Mannering *et al.* (10) have recently observed a dietary essential corresponding to GPF-3 in liver fraction L, and have noted some of these properties.

EXPERIMENTAL

The general procedure in experiments with guinea pigs was the same as described earlier (1).

Optimal Casein Level—To determine the optimal level of casein in the ration the following diet was used: glucose 52 parts, Cellu flour 20 parts, salts (11) 5 parts, liver fraction L 5 parts, vitamins A, D, E, K, corn oil (1) 1 part, the crystalline vitamins as used previously (1), and casein (Labco vitamin-free) 18 parts. When the amount of casein was increased, the amount of glucose was decreased correspondingly. From the data in Table I it can be seen that optimal growth was obtained when the ration contained 30 per cent of casein. 40 per cent was harmful. Furthermore, a mixture of arginine, cystine, and glycine produced the same effect as the extra casein.

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[2] This fraction was the alcohol-insoluble portion of liver extract rendered water-soluble. We wish to thank Dr David Klein of The Wilson Laboratories for gifts of this material

[3] Folic acid is used in this paper to denote the substance or substances which promote growth of *Lactobacillus casei* under conditions similar to those described by Snell and Peterson (6) and Landy and Dicken (7)

TABLE I

Effect of Various Levels of Casein and of Amino Acids on Growth and Survival of Guinea Pigs

Casein	Arginine	Cystine	Glycine	No of animals	No of deaths	Average change in weight
per cent	*per cent*	*per cent*	*per cent*			*gm per wk.*
18				11	0	12
25				4	0	19
30				10	0	27
40				4	1	11
18	1.0	0.1	0.2	7	0	27

TABLE II

Effect of Insoluble Materials on Growth and Survival of Guinea Pigs

Materials	Amount in ration	No of animals	No of deaths	Average change in weight of survivors
	per cent			*gm. per wk.*
None		16	3	19
Cellu flour	20	12	0	28
Powdered cellophane	20	4	0	26
Silica gel*	20	8	4	28
" "	10	4	1	10
Super-Cel†	20	4	1	20
Powdered asbestos	20	4	1	14
Agar	20	4	3	
Norit	20	4	4	

* The Davison Chemical Corporation.

† Johns-Manville Corporation.

Effect of Cellulose—In order to study the effects produced by various fibrous materials the following ration was used: glucose 57 parts, casein (Labco vitamin-free) 30 parts, salts, oils, and vitamins as above, and liver fraction L 8 parts. The materials to be tested were added to the ration at the expense of some of the glucose. Data illustrating the responses for a 4 week interval are shown in Table II. Cellulose supplied as Cellu flour or cellophane was the only material that was active. Some of the substances were decidedly deleterious.

Preparation of GPF-3 Concentrate Low in Folic Acid—500 gm. of liver fraction L were dissolved in 10 liters of water and the solution was treated with 625 cc. of 20 per cent lead acetate solution. The precipitate was filtered off, washed with water, and discarded. After the lead was removed as PbS, the filtrate was made to pH 3.4 with HCl and stirred with 200 gm. of norit A for 1 hour The norit adsorbate was filtered off, washed with water, and discarded. The filtrate was concentrated by evaporation, neutralized with NaOH, and concentrated further to 500 cc. This preparation was called lead acetate-norit filtrate (LAN). When assayed for folic acid by the use of *Lactobacillus casei* (7), it was found to contain 0.45 γ of folic acid per gm. of liver fraction L taken, while the liver extract from which it was made contained 9 γ per gm.

Folic Acid Deficiency in Guinea Pigs—In order to study folic acid deficiency in guinea pigs the following basal ration was used: glucose 37 parts, casein (Labco vitamin-free) 30 parts, salts, oils, and vitamins as used above, Cellu flour 20 parts, and the above LAN preparation equivalent to 8 parts of liver fraction L. When fed this basal ration young guinea pigs stopped growing usually after about 3 weeks. The weight remained more or less stationary for a week or two, and then a precipitous loss ensued, followed by death. During the period of weight loss the animals became extremely emaciated and lethargic. They would stand quietly in a hunched position for considerable periods of time. A few days before death a gentle shove frequently would throw the animal on its side. The terminal stage before death was nearly always a convulsion in which the animal would fall on its side and twitch its head and legs spasmodically. Salivation was occasionally profuse.

When liver fraction L replaced the LAN preparation in the above basal ration, good growth and apparently normal health were maintained. Similarly, when a folic acid concentrate prepared from liver fraction L (12) was added to the basal ration, the animals remained healthy and grew well. Finally, when crystalline folic acid in the form isolated by Stokstad (13) or that isolated by Pfiffner *et al.* (14) was added, good growth resulted.[4] The experiments lasted 4 to 5 weeks. Due to lack of material, animals receiving the crystalline folic acid preparations were fed only the basal ration for the first 2 weeks. The data are summarized in Table III. Some of these data were presented in a preliminary publication (15), but are included in Table III for the sake of completeness. When folic acid concentrate was added to the basal ration and the LAN preparation was omitted, failure of the animals occurred, due to the deficiency of GPF-3.

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[4] Although both forms of crystalline folic acid were active for guinea pigs, it should be noted that they differ quantitatively in activity for *Lactobacillus casei* and *Streptococcus lactis* R (13). We wish to thank Dr. E. L. R. Stokstad and Dr. B. L. Hutchings of the Lederle Laboratories, Inc., and Dr. A. D. Emmett of Parke, Davis and Company for gifts of these materials.

Assay Procedure for GPF-3—In order to test preparations for GPF-3 activity the basal ration used to demonstrate folic acid deficiency was employed with the following changes. The LAN preparation was replaced by an equal weight of glucose and the animals were given 6 γ of folic acid per day by pipette. In these experiments the concentrate of folic acid (12) rather than the crystalline material was used. On this régime guinea pigs grew poorly and survived for approximately 4 weeks. When preparations containing GPF-3 were added, growth was quite satisfactory and the animals remained in good health throughout the assay period of 5 weeks.

TABLE III

Effect of Folic Acid Preparations on Growth and Survival of Guinea Pigs

Preparation	Folic acid intake	No. of animals	No. of deaths	Average change in weight of survivors
	γ per day			*gm per wk.*
None (basal ration)	0.5	12	12	
Liver fraction L	10	17	0	25
Folic acid concentrate	13.5	11	0	28
Crystalline folic acid (Stokstad)	6.5	2	0	23
" " " (Pfiffner *et al.*)	6.5	2	0	19

Concentration of GPF-3—The LAN preparation described above was as active as any obtained thus far. A preparation of about the same potency was made by extraction of the finely powdered liver fraction L with 10 volumes of alcohol to which was added enough concentrated HCl to give a final pH of 2.3. The extract was freed of alcohol under reduced pressure, neutralized, and assayed.

The GPF-3 activity of liver fraction L was relatively stable to alkali. Thus, when the material was adjusted to pH 10 with NaOH and the solution treated with enough additional NaOH to make it 1 N, refluxed for 4 hours, and neutralized, the activity still remained. However, some preliminary experiments have indicated that the activity of more purified material was lost in alkali. Data on the growth of guinea pigs fed the above concentrates are shown in Table IV.

Effect of Biotin and p-Aminobenzoic Acid—When biotin and *p*-aminobenzoic acid were added to the basal ration used for the assay of GPF-3, the survival time of the animals was increased several weeks. Thus of thirteen pigs fed 1 γ of biotin and 100 γ of *p*-aminobenzoic acid per day, all survived for 6 weeks but only five were alive at the end of the 9th week. Control animals receiving no biotin and no *p*-aminobenzoic acid were all dead in 5 weeks. The average change in weight (17 gm. per week) during the first weeks of the experiment was not significantly different in the two groups. Nine of ten positive controls which received liver fraction L survived for 10 weeks and gained an average of 25 gm. per week. In later assays for GPF-3, therefore, biotin and *p*-aminobenzoic acid were fed.

TABLE IV

Effect of GPF-3 Preparations on Growth and Survival of Guinea Pigs

Preparation	Amount in ration	No of animals	No. of deaths	Average change in weight of survivors
	per cent			*gm. per wk*
None (basal ration)		12	11	
Liver fraction L	8	23	0	26
Lead acetate-norit filtrate	≈8	11	0	28
Alcoholic HCl extract	≈8	4	0	21
NaOH digest	≈8	3	0	23

SUMMARY

The dietary essential, GPF-2, previously demonstrated to be required by guinea pigs, was replaceable by a mixture of cellulose and casein or cellulose, arginine, cystine, and glycine. The optimal amount of casein for growth of guinea pigs fed a highly purified ration was found to be 30 per cent of the diet. The dietary essential, GPF-1, was identified with folic acid, and the behavior of guinea pigs on a folic acid-deficient ration was described. A highly purified diet for the assay of the unidentified nutritional essential GPF-3 was developed. Some improvement in survival time of GPF-3-deficient animals was observed when biotin and *p*-aminobenzoic acid were fed. It was shown that GPF-3 was not precipitated by lead acetate, was not readily adsorbed by norit, and that while it was insoluble in alcohol it was soluble in alcohol and HCl. It resembled strepogenin, an unidentified growth factor required by certain bacteria.

BIBLIOGRAPHY

1. Woolley, D W., *J. Biol. Chem* , **143**, 679 (1942)
2. Sober, H. A., Mannering, G. J., Cannon, M D , Elvehjem, C. A., and Hart, E. B., *J. Nutr* , **24**, 503 (1942).
3. Hogan, A. G., and Hamilton, J. W., *J. Nutr*, **23**, 533 (1942)

4. Hegsted, D. M., Briggs, G. M., Elvehjem, C. A., and Hart, E. B , *J. Biol Chem.*, **140**, 191 (1941).
5. Zucker, T., and Zucker, L., *Proc. Soc. Exp. Biol. and Med.*, **55**, 136 (1944).
6. Snell, E. E., and Peterson, W. H., *J. Bact.*, **39**, 273 (1940).
7. Landy, M., and Dicken, D. M., *J. Lab. and Clin. Med.*, **27**, 1086 (1942).
8. Woolley, D. W., *J. Exp. Med.*, **73**, 487 (1941).
9. Sprince, H., and Woolley, D W , *J. Exp. Med* , **80**, 213 (1944).
10. Mannering, G. J., Cannon, M. D , Barki, H. V., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, **151**, 101 (1943).
11. Phillips, P. H., and Hart, E. B , *J. Biol. Chem.*, **109**, 657 (1935).
12. Hutchings, B. L., Bohonos, N., and Peterson, W. H., *J. Biol. Chem.*, **141**, 521 (1941).
13. Stokstad, E. L. R., *J. Biol. Chem.*, **149**, 573 (1943).
14. Pfiffner, J. J., Binkley, S. B , Bloom, E S., Brown, R. A., Bird, O. D., Emmett, A. D., Hogan, A. G., and O'Dell, B. L , *Science*, **97**, 404 (1943).
15. Woolley, D. W., and Sprince, H , *J. Biol Chem.*; **153**, 687 (1944).

PRODUCTION OF NICOTINIC ACID DEFICIENCY WITH 3-ACETYLPYRIDINE, THE KETONE ANALOGUE OF NICOTINIC ACID

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Since it has been shown during the past two years that diseases with signs characteristic of specific vitamin deficiencies may be produced by the feeding of certain structural analogues of various vitamins, and that these diseases may be cured by adequate doses of the vitamin concerned (1–4), it has seemed desirable to study the types of structural change which, when applied to a vitamin or other metabolite, will result in the formation of antagonistic agents. Although the substitution of sulfonic acid or sulfonamide groups for the carboxyl groups of acidic vitamins has given rise to compounds (*e.g.* sulfonilamide (5), thiopanic acid (6, 7), and pyridine-3-sulfonic acid (8)) that cause specific vitamin deficiencies of certain microorganisms, this type of change has not been very successful for the formation of agents capable of producing vitamin deficiency diseases in animals (9). Therefore, attempts have been made to learn what alterations of the carboxyl group will result in the realization of antagonistic analogues which are effective in animals.

Two previous observations led to the finding that 3-acetylpyridine will cause nicotinic acid deficiency in animals. In 1938 Woolley *et al.* (10) found that dogs suffering from nicotinic acid deficiency were promptly killed by a single dose of 3-acetylpyridine, while normal dogs were unharmed by similar doses of the compound. In 1942 Auhagen (11) reported that *p*-aminoacetophenone was bacteriostatic, and that this action was reversed by *p*-aminobenzoic acid. These facts indicated that the exchange of $—COCH_3$ for $—COOH$ might be a general method for the formation of inhibitory analogues. The observations in this paper lend support to such a suggestion, but more cases will be required to establish this type of structural change as one of general applicability.

3-Acetylpyridine was observed to cause a disease in mice characterized by many of the signs seen in nicotinic acid-deficient dogs and humans. This disease was very rapid in onset following administration of the drug. When sufficient quantities of nicotinic acid or nicotinamide were given, the signs of the disease did not appear. This fact indicated that the signs resulted from a deficiency of nicotinic acid or nicotinamide in the animal. It was of interest to note that despite these reactions in the animal, 3-acetylpyridine did not inhibit the growth of microorganisms in a manner subject to specific reversal by nicotinic acid. Auhagen (11) observed results similar to these with bacteria.

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* With the technical assistance of M. L. Collyer.

EXPERIMENTAL

3-Acetylpyridine was synthesized according to the directions of Strong and McElvain (12).

Production of Disease of Mice with 3-Acetylpyridine—Weanling mice (10 to 12 gm.) were caged on screen floors and fed a ration composed of sucrose 75 gm., vitamin-free casein (Labco) 18 gm., salts (13) 5 gm., fortified corn oil (14) 1 gm., thiamine 0.2 mg., riboflavin 0.5 mg., pyridoxine 0.2 mg., calcium pantothenate 2 mg., choline chloride 100 mg., and inositol 100 mg. Since mice grew as rapidly on this ration as on the ration plus nicotinic acid (15), it was concluded that mice, as do rats, synthesized their own supply of nicotinic acid. 3 days after the start of the tests, 3-acetylpyridine was given orally in single daily doses.

The signs of the disease which resulted were in some degree dependent on the size of the daily dose. When more than 10 mg. per mouse per day were given, the animals exhibited rapid respiratory rate, soon followed by loss of control of the hind legs, and death within the 1st day. The best sequence of events was seen with 2 to 4 mg. doses, for this amount allowed the signs to develop for a number of days before death ensued. Very soon after the first dose the animals began to breathe quite rapidly. In a few hours difficulties in control of the hind legs were seen. Occasionally an animal would stand upright in the manner of a begging dog. Within 2 days almost complete paralysis of the hind legs resulted. At about the same time the mice appeared extremely wet and unkempt. Emaciation was usually prominent. The skin, first on the chest wall, and then on the sides and legs, became very red and inflamed. Fiery red tongues did not develop until the 4th to 7th day, and then they appeared in only about half of the animals. It was of interest that these red tongues were never seen in animals given sufficient drug to cause fatal disease in 1 or 2 days, but that a relatively long period of onset was necessary for their appearance.

Prevention of Disease with Nicotinic Acid or Nicotinamide—When sufficient nicotinic acid or nicotinamide was added to the ration, the signs of the disease were prevented, and the mice grew at a rate comparable to that seen in animals getting no 3-acetylpyridine. It was found advisable to feed the rations containing nicotinic acid or nicotinamide for 3 or 4 days before the drug was administered. If this was not done the animals frequently had not begun to eat well, and hence were not sufficiently fortified to withstand the effects of the 3-acetylpyridine. Only partial success was had in attempts to save animals ill from the drug by oral administration of relatively large doses of ethyl nicotinate, although this ester was effective in prevention of the disease if its use was begun 3 days before the 3-acetylpyridine was given. Some representative data on the responses of mice to 3-acetylpyridine and to this compound plus nicotinic acid or nicotinamide are shown in Table I. Whenever nicotinic acid was used, enough sodium bicarbonate was added to the ration to neutralize the acid.

Effect of 3-Acetylpyridine on Microbial Growth—3-Acetylpyridine inhibited the growth of *Lactobacillus casei* cultured in the nicotinic acid-free medium of Landy and Dicken (16). Half maximal inhibition of growth was produced by 2 mg. of the compound per cc. Small amounts of nicotinic acid did not diminish the inhibition of growth caused by 2 mg. of 3-acetylpyridine per cc., but as the concentration of nicotinic acid was raised above 200 γ per cc. the inhibition of growth was reversed. When the concentration of 3-acetylpyridine was doubled, the quantity of nicotinic acid necessary for reversal likewise was doubled. However, this antagonistic action of nicotinic acid was a non-specific one because it was likewise produced by acetic acid and because the effect was not produced by nicotinamide or by sodium nicotinate.

TABLE I

Effect of 3-Acetylpyridine and 3-Acetylpyridine Plus Nicotinic Acid on Growth and Survival of Mice

3-Acetyl-pyridine	Nicotinic acid	Nicotinamide	Animals	Deaths	Average change in weight	Survival time
mg. per day	*per cent of ration*	*per cent of ration*			*gm. per wk.*	*days*
0	0	0	10	0	+3.5	>14
10	0	0	19	19		1
4	0	0	24	21		3
2	0	0	4	1		4*
1	0	0	10	0	+1.0	>9
10	2.0	0	3	0	+3.8	>7
10	0.2	0	6	4		1-3*
4	2.0	0	10	0	+3.0	>14
4	0.2	0	10	0†	+2.2	>14
4	0	0.5	9	0	+1.9	>7

* Survival time of the animals that died.

† One of the mice developed redness of the skin on the ventral surface, and unkempt hair.

3-Acetylpyridine was even less active against *Saccharomyces cerevisiae*, *Escherichia coli*, *Staphylococcus aureus*, and *Lactobacillus arabinosus* than against *Lactobacillus casei*. No detectable effect was observed when the last two organisms were cultivated in the presence of 4 mg. of the ketone per cc., and the slight inhibition of growth of the first two species which resulted from large doses of the compound was not reversed by nicotinic acid.

Effect of Growing Cultures of Lactobacillus arabinosus on 3-Acetylpyridine—To determine whether the resistance of microorganisms to the action of 3-acetylpyridine was due to the ability of such species to inactivate the compound, the following experiment was performed. *Lactobacillus arabinosus* was grown at 30° for 48 hours in the medium of Landy and Dicken (16) plus 4 mg. of 3-acetylpyridine per cc. The luxuriant crop of organisms was filtered off and the filtrate was made alkaline and extracted four times with chloroform. The chloroform was removed from the extract and the resulting residue was assayed on mice. When it was fed to each of six mice at a level equivalent to 10 mg. of the 3-acetylpyridine originally added to the culture medium, five of the six exhibited characteristic signs and died in 20 to 40 hours. Six mice which were fed the basal ration plus 0.5 per cent of sodium nicotinate remained well when they were given the same amount of the culture extract as was used for the former animals. It was therefore concluded that the majority of the 3-acetylpyridine was unchanged by the growing bacteria.

DISCUSSION

From the foregoing experiments it appears that 3-acetylpyridine is an effective agent for causing signs of nicotinic acid deficiency, even in species such as the mouse for which nicotinic acid is not a dietary essential. In this respect it resembles glucoascorbic acid, an analogue of vitamin C which allows the production of a scurvy-like disease in animals which do not require ascorbic acid in the diet (2). If nicotinic acid and ascorbic acid are regarded as hormones in species such as the mouse (and they are certainly not vitamins for this species), then 3-acetylpyridine and glucoascorbic acid may be viewed as compounds with structures analogous to, but with actions antagonistic to, hormones. With 3-acetylpyridine it may be possible to study some of the manifestations of nicotinic acid deficiency in species where this would otherwise be impossible.

In view of the action of 3-acetylpyridine in causing signs of nicotinic acid deficiency in animals, its ineffectiveness against bacteria is puzzling. Even against microbial species for which nicotinic acid is an essential growth factor it is relatively inert. Furthermore, this is not due to any ability of the bacteria to inactivate the substance. Either the microorganisms have effective means of preventing the entry of this harmful compound, or they lack those metabolic reactions involving nicotinic acid with which 3-acetylpyridine interferes in the animal organism. If either of these possibilities is correct, an elucidation of the process involved would be illuminating.

The contrast in the action of 3-acetylpyridine and pyridine-3-sulfonic acid is noteworthy. The former is effective against mice and ineffective against microorganisms, while for the latter the reverse is true (9).

SUMMARY

Typical signs of nicotinic acid deficiency as seen in susceptible species were caused by 3-acetylpyridine when 2 or more mg. per day were fed to mice. This species was not susceptible to nicotinic acid deficiency produced by the usual dietary means. The signs of the disease were prevented by sufficient amounts of nicotinic acid or of nicotinamide in the ration. 3-Acetylpyridine was regarded as the structural analogue of nicotinic acid in which the —COOH group of the vitamin had been exchanged for —$COCH_3$. In contrast to the results with animals, 3-acetylpyridine was relatively ineffective for the inhibition of growth of microbial species, and in those instances in which inhibition of growth was produced it was not reversed specifically by nicotinic acid. This ineffectiveness against bacteria was not due to the ability of these organisms to inactivate the compound.

BIBLIOGRAPHY

1. Woolley, D W , and White, A. G. C , *J. Biol. Chem* , **149,** 285 (1943)
2. Woolley, D W , and Krampitz, L O , *J Exp Med* , **78,** 333 (1943).
3. Woolley, D. W , *J Biol Chem* , **154,** 31 (1944).
4. Emerson, G A , and Tishler, M , *Proc Soc. Exp. Biol and Med* , **55,** 184 (1944)
5. Woods, D D , *Brit J Exp Path* , **21,** 74 (1940).
6. Snell, E E , *J. Biol. Chem* , **139,** 975 (1941).
7. McIlwain, H , *Biochem J* , **36,** 417 (1942)
8. McIlwain, H , *Brit J Exp. Path* , **21,** 136 (1940)
9. Woolley, D W , and White, A G C , *Proc Soc Exp Biol and Med.*, **52,** 106 (1943).
10. Woolley, D W , Strong, F. M., Madden, R J , and Elvehjem, C A., *J. Biol. Chem* , **124,** 715 (1938)
11. Auhagen, E , *Z physiol Chem* , **274,** 48 (1942)
12. Strong, F M , and McElvain, S. M , *J Am Chem. Soc.*, **55,** 816 (1933).
13. Phillips, P. H., and Hart, E. B., *J. Biol. Chem* , **109,** 657 (1935).
14. Woolley, D. W , *J. Biol Chem* , **143,** 679 (1942)
15. Woolley, D. W , *Proc. Soc Exp Biol and Med* , **46,** 565 (1941)
16. Landy, M., and Dicken, D. M , *J Lab. and Clin Med* , **27,** 1086 (1942).

BLOOD AS A PHYSICOCHEMICAL SYSTEM

XIII. THE DISTRIBUTION OF LACTATE

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For some physicochemical purposes the assumption suffices that in the blood of men at rest chloride, bicarbonate, and proteinate are the only quantitatively important anions (9). In exercise the concentration of lactate may approach or exceed that of bicarbonate. This fact led us to choose for study systems containing lactate in concentrations representative of those observed after severe muscular exertion, the purposes being, first, to see whether lactate behaves like chloride with changing partial pressures of oxygen and of carbon dioxide and, second, to analyze the kinetics of the movement of lactate from plasma into red cells.

Three main conclusions will be developed below. First, when plasma and cells are in equilibrium, lactate can be treated as a physicochemical component of blood in the sense used by Henderson (9). Second, the rate of attaining equilibrium when lactate moves into red cells is slow enough to allow analysis of the kinetics of the system, and during the attainment of equilibrium the movements of lactate, chloride, and bicarbonate are related to one another. Third, the rates of transfer of chloride and bicarbonate are conditioned by the rate of transfer of lactate. After describing the analytical methods used, we shall discuss these findings in order.

Methods

The symbols to be used throughout this paper are those of Henderson (9), with the following additions: L_{WB} is the concentration of lactate in milliequivalents per liter of whole blood; L_c is the concentration of lactate in milliequivalents per liter of red cells; L_p is the concentration of lactate in milliequivalents per liter of plasma; r_L is the Gibbs-Donnan distribution ratio for lactate, $(L_c \times (H_2O)_p)/(L_p \times (H_2O)_c)$.

Lactate was estimated by the method of Edwards (5); blood CO_2 and O_2 by methods described before (9, 15); chloride by the method of Van Slyke and Sendroy (16); water by drying to constant weight at 110°; pH with the glass electrode as described by Dill, Daly, and Forbes (2); cell volume by the centrifuge method of Hirota (10), in which three readings are taken and the equilibrium value is obtained by extrapolation; and protein by a micro-Kjeldahl method (12).

The experimental techniques were based on six chief considerations: (*a*) L_c can be calculated reasonably accurately from L_{WB}, L_p, and V_c (Table

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* Died, December 14, 1937.

I) either for high or low concentrations of lactate. (*b*) Once lactic acid has been added to whole blood, V_c does not change either with change in temperature or with change in L_c/L_p (Table II). (*c*) In the mixing apparatus to be described below, cooling of the blood from 37–0° can be accomplished in not more than 20 seconds. (*d*) In the same apparatus plasma can be mixed intimately with cells in not more than 30 seconds. (*e*) The only difference in L_c/L_p in the temperature range 0°–39° that matters for present purposes is the difference in rate of attaining equilibrium, not in the final ratio, which is the same at any temperature (Fig. 1). (*f*) After plasma and cells are mixed, L_p changes so slowly at 0° (Fig. 1), even when it is very large in comparison with L_c, that it may be regarded as constant for periods as long as an hour.

TABLE I

Agreement between Values for Cell Lactate As Calculated and As Estimated Directly

Sample No	L_{WB}	L_p	L_c	
			Estimated directly	Calculated
	m eq. per l.	*m eq per l*	*m eq per l*	*m eq. per l.*
1	7.90	12.10	2.94	2.94
12	11.23	14.57	6.76	7.05
58	2.13	2.52	1.38	1.65

TABLE II

Constancy of V_c with Changing Temperature and L_c/L_p

Experiment No.		L_{WB}	Temperature	$\frac{L_c}{L_p}$	V_c
		m eq per l	°*C*		*ml per 100 ml.*
58	Blood as drawn	1.9	39	0.59	45.4
	Added lactate	10.5	0	0.15	48.8
	" "	10.4	38	0.37	48.8
59	" "	17.7	0	0.06	51.8
	" "	19.6	38	1.25	51.7
63	" "	12.7	0	0.11	47.5
	" "	12.4	28	0.37	47.3
	" "	13.5	28	0.55	47.3
67	" "	11.1	0	0.07	46.1
	" "	11.3	23.5	0.34	46.8
	" "	11.6	23.5	0.41	47.0

When lactate was studied in relation to the physicochemical system hemoglobin, plasma protein, oxygen, carbon dioxide, hydrogen ion, chloride, and bicarbonate, venous blood of high lactate concentration was drawn from subjects who had just run vigorously to exhaustion. It was heparinized, equilibrated in tonometers at the desired pCO_2 and pO_2, and analyzed by the techniques of Henderson and associates (9).

When rates of diffusion were studied, venous blood was drawn into heparin under oil at about 4°. Plasma was separated by centrifuging and was drawn off under oil, and pure crystalline *l*(+)-lactic acid was added to it in amounts approximating those found after severe muscular exercise.

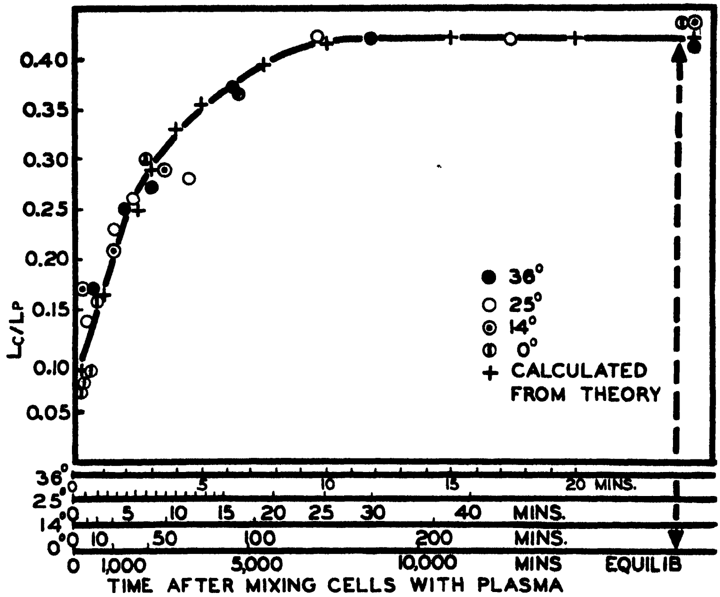

FIG 1. Rate of diffusion of lactate into cells at various temperatures Ordinates, ratio of lactate concentration in cells and in plasma; abscissae, time after mixing cells and plasma. Abscissae at different temperatures adjusted to show similarity of curves. Calculated points derived as described in the text.

The centrifuge tube with its contained cells was filled with oil, placed in a constant temperature bath, and fitted with a rubber stopper carrying three glass tubes. The first connected with a reservoir of mercury so that pressure could be adjusted in the centrifuge tube by raising or lowering the mercury. The second led a stainless steel stirring rod into the cells through an air-tight oiled bushing. Stirring was by means of an electric motor connected to the stirring rod and run continuously throughout all experiments. The third hole connected with a 2-way glass stop-cock, the glass tubing of which extended to the bottom of the centrifuge tube. The plasma, which had been kept at about 4° during the above manipulations, was now brought to the same temperature as the cells, and the subject's blood was reconstituted by sucking the plasma back into the centrifuge tube through the 2-way stop-cock under a negative pressure produced by lowering the mercury reservoir. At any desired time samples of reconstituted blood could be forced under oil out of the 2-way stop-cock into small centrifuge tubes surrounded by ice. All subsequent manipulation of these samples was at about 0°. Whole blood and hematocrit samples were taken at the beginning and end of the experiments. Plasma was obtained by centrifuging for 10 minutes at about 4°.

When the interdependent relations of lactate and chloride at equilibrium were under investigation, venous blood was drawn into heparin under oil, the cells were centrifuged down at the desired temperature, the plasma was withdrawn under oil, mixed with the desired amount of a 40 per cent aqueous solution of pure crystalline $l(+)$-lactic acid,[1] and remixed with the cells at the desired temperature, and samples of this reconstituted blood were withdrawn after equilibrium was established. Stirring was manual and intermittent.

Results

Lactate As Component of Physicochemical Systems at Equilibrium—The general conclusions from experiments on systems containing lactate in high concentrations (Table III) are that lactate behaves like chloride and both substances may be treated as physicochemical components of the system by the same theoretical considerations. In addition, the Henderson-Hasselbalch equation appears to be valid up to about pH 7.2 for such a system, as judged by the reasonably good agreement between the calculated pH and the pH as measured with the glass electrode. Above pH 7.2 the observed values are lower than the calculated for undetermined reasons. The same phenomenon is observed in systems low in lactate.

The Gibbs-Donnan ratio for chloride, bicarbonate, and lactate displays certain points of interest in systems whose lactate concentration is high (Fig. 2). In comparison with the blood of man at rest (3, 17), r_{Cl} is depressed and r_{HCO_3} is increased; r_L is similar to r_{Cl} in magnitude but somewhat smaller. This phenomenon is seen in Table IV where the same original sample of blood was studied with and without added lactic acid.

Rate of Diffusion of Lactate from Plasma into Cells; Systems at Fixed Temperature—The rate of diffusion of lactate from plasma into cells at any physiological temperature is easily measured, since even at 37° the time necessary to attain one-half the equilibrium value when lactic acid is added to plasma is about 2 minutes, while at 0° it is about 20 hours. By rapid

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[1] We are indebted to Professor J. Yule Bogue, Royal Veterinary College, London, England, for supplying us with this lactic acid.

TABLE III

Physicochemical System in Blood of F. C.

Experiment No	Tonometer No	HbO_2 capacity	HbO_2 content	$(CO_2)_{WB}$	pCO_2	V_c	$(H_2O)_c$	$(H_2O)_p$	$(Protein)_p$	$(Proteinate)_c$	$(Proteinate)_p$	$(HCO_3)_c$	$(HCO_3)_p$	$(Cl)_c$	$(Cl)_p$	$(Lactate)_c$	$(Lactate)_p$	$(pH)_s$ observed	$(pH)_s$ calculated	r HCO_3	r Cl	r L
		mM per l	*mM per l*	*mM per l*	*mm Hg*	*ml per l*	*gm per l*	*gm per l.*	*gm per l*	*m eq per l*	*m eq per l*	*m eq per l*	*m eq per l*	*m eq. per l*	*m eq per l*	*m eq per l*	*m eq per l*					
1. Reduced	1	9.04	0.44	1.87	2 9	468	723	935	71.6	27.9	16 8	1.4	2.1	60.2	105.5	10.9	23.3	7.34	7.47	0.87	0.74	0.60
	2	9.04	0 35	10.15	32 6	487	727	933	73.3	17.3	15 3	8 5	9.8	64.1	103.5	12.7	21.8		7.09	1.11	0.79	0.75
	3	9.04	0.27	26.51	194.5	493	740	932	74.9	−4.3	12 4	19.5	22.2	70.1	97.5	13.7	21.0		6.67	1.11	0.91	0.82
Oxygenated	1	9.04	9.00	2.20	4.5	460	713	935	71.1	31.3	15.7	2.1	2.1	56 8	107 9	11.3	23.1	7 20	7.28	1.31	0.69	0.64
	2	9.04	9.04	10.07	43 4	472	728	934	72 1	19.2	14 2	7.5	10.0	62.8	104.4	11.7	22.2		6.97	0.96	0.77	0.68
	3	9.04	9.08	25.71	199 5	481	732	933	73.6	0.4	11 9	19.4	20.5	68.8	99.4	13.3	21.0		6.63	1.20	0.88	0.80
2. Reduced	1	8.85	0.26	3.50	4.2	448	716	934	71.7	35.0	17 9	2.9	3.8	53.5	108.5	7.9	16.4	7.48	7.57	0.99	0.64	0.63
	2	8.85	0.31	13 26	33.3	456	730	933	73.4	22.1	16.3	11.2	13.4	57.9	103.9	8.7	15.7	7.22	7.22	1.07	0.71	0.71
	3	8.85	0.37	31.20	199.5	465	739	931	75.2	−0.1	13 0	23.9	27.0	63.3	99.2	8.8	15.2	6.74	6.74	1.12	0.80	0.73
Oxygenated	1	8.85	8.89	3.23	5 6	442	720	934	71.4	38.1	16.7	2.3	3.8	48.9	110.1	6.7	16.7	7.33	7.45	0.79	0.58	0.52
	2	8.85	8.80	11 99	37.8	456	726	933	73.3	27.0	15.6	9.1	12.5	57.7	105.0	7.8	16.1	7.10	7.13	0.93	0.70	0.62
	3	8.85		28 13	186.5	462	734	932	74 3	0.6	12.8	20.8	24.6	62 1	99.5	8.9	14.9		6.74	1.08	0.79	0.76

cooling of the blood to about 0°, the movement of lactate is in effect stopped, and thus the state of the system at any given time can be determined.

If the cell membrane is assumed to be freely permeable in both directions to lactate, then the rate of diffusion from plasma to cells when the gradient is in that direction can be expressed by an equation derived as follows: In addition to the symbols defined above, *A* is the area of cells; *h* is the thickness of the cell membrane; *k* is the diffusion constant; *t* is the time in minutes after mixing cells and plasma; and V_c is the cell volume expressed as a fraction of 1. Assuming Fick's principle (6),

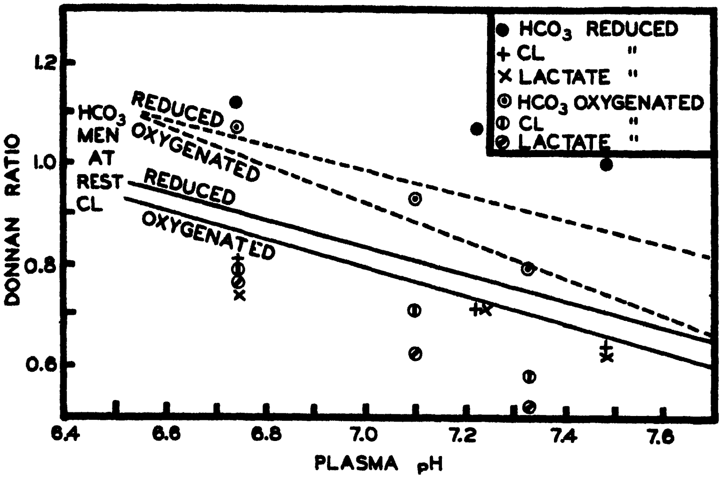

FIG. 2. Distribution ratios of bicarbonate, chloride, and lactate at 37° in blood of subject S. Blood high in lactate compared with usual results for blood of man at rest. Dotted lines, values for bicarbonate in man at rest, and unbroken lines, values for chloride in man at rest, both from Dill, Edwards, and Consolazio (3). The points for blood high in lactate are not connected by lines. Abscissae, pH_s; ordinates, Gibbs-Donnan ratio.

$$\frac{d(L_p)}{dt} = -k\frac{A}{h}(L_p - L_c)$$

Since in the present system V_c and A do not change and h is unknown, let $k(A/h)$ represent the "permeability constant" K of Jacobs and Stewart (11). It is of the dimensions milliequivalents per minute.

Now

$$L_{WB} = L_c V_c + L_p(1 - V_c)$$

and

$$L_c = \frac{L_{WB} - L_p(1 - V_c)}{V_c}$$

TABLE IV

Diffusion of Chloride and Bicarbonate in Relation to Lactate

Measurement	0°						37°					
	No added lactate			Added lactate			No added lactate			Added lactate		
	Blood	Plasma	Cells*	Blood	Plasma	Cells*	Blood	Plasma	Cells*	Blood	Plasma	Cells*
V_c, *ml. per 100 ml.*	47.5			47.8			47.0			47.3		
H_2O, *gm. per l.*	834	948	709	832	942	714	834	948	706	831	942	710
O_2 content, *ml per 100 ml.*	10.38			11.24			10.22			11.44		
" capacity, " " *100* "	22.70			22.90			22.43			22.63		
CO_2 content, *ml. per 100 ml.*	60.6	72 4	47.8	60 2	64.5	55.6	61.4	72.8	48.5	60.3	67.4	52.4
pH, glass electrode		7.33			6.97			7.31			6.98	
Lactate, *m.eq. per l.*	1.9	2.5	1.3	14.0	25 0	1.9	2.7	4 0	1.3	14.0	19.8	7.6
Chloride, " " "	79.5	100.1	56.7	80.2	93.5	65.7	78.4	100.3	53.6	80.0	98.0	59.8
Bicarbonate, *m.eq. per l.*	25.4	30.6	19.5	23.7	25.5	21.7	25.9	30.7	20.4	23.9	26.7	20.6
r_L	0.69			0.10			0.44			0.51		
r_{Cl}	0.76			0.93			0.72			0.81		
r_{HCO_3}	0.85			1.12			0 89			1.02		

* Calculated from V_c, value for whole blood and value for plasma.

substituting in the first equation, we obtain,

$$\frac{d(\mathrm{L}_p)}{dt} = -K\left(\frac{\mathrm{L}_p V_c - \mathrm{L}_{WB} + \mathrm{L}_p - \mathrm{L}_p V_c}{V_c}\right) = -\frac{K}{V_c}(\mathrm{L}_p - \mathrm{L}_{WB})$$

$$\frac{d(\mathrm{L}_p)}{\mathrm{L}_p - \mathrm{L}_{WB}} = -\frac{K}{V_c}\,dt$$

On integration,

$$\mathrm{L}_p - \mathrm{L}_{WB} = be^{-Kt/V_c} \quad \text{and} \quad \mathrm{L}_p = \mathrm{L}_{WB} + be^{-Kt/V_c}$$

By putting $t = 0$, it is seen that $b = (\mathrm{L}_{p_0} - \mathrm{L}_{c_0})V_c$ and is therefore the measure of the initial lactate gradient. By steps similar to the derivation of L_p,

$$\mathrm{L}_c = \mathrm{L}_{WB} - \left(\frac{1 - V_c}{V_c}\right)be^{-Kt/V_c}$$

Owing presumably to Gibbs-Donnan effects, the concentration of lactate at equilibrium is never the same in cells and in plasma as it should be at $t = \infty$ if the system obeyed the laws of simple diffusion. Hence, it is necessary to insert arbitrary constants E_p and E_c to correct for this aberration, making

$$\mathrm{L}_p = E_p[\mathrm{L}_{WB} + be^{-Kt/V_c}]$$

and

$$\mathrm{L}_c = E_c\left[\mathrm{L}_{WB} - \left(\frac{1 - V_c}{V_c}\right)be^{-Kt/V_c}\right]$$

If L_c is divided by L_p,

$$\mathrm{L}_c/\mathrm{L}_p = E\left(\frac{\mathrm{L}_{WB} - \frac{1 - V_c}{V_c}be^{-Kt/V_c}}{\mathrm{L}_{WB} + be^{-Kt/V_c}}\right)$$

The calculated curves fit the observed curves tolerably well, as is shown in Table V and Fig. 1 for two different experiments. Typical values for E are 0.4 to 0.6; for b, 0.4 to 7.0; and K is variable according to the temperature, as discussed below.

Attempts to measure the rate of diffusion from cells of high lactate content into plasma of low lactate content have been unsatisfactory because under our experimental conditions gross hemolysis has always occurred when the plasma and cells were mixed.

Effects of Temperature on Cell Permeability—Change of temperature has a striking effect on the permeability of red cells by lactate. As the temperature decreases in blood, the "permeability constant" for lactate becomes smaller much more abruptly than it would if it obeyed an equation of the Arrhenius type, in which Q_{10} is relatively constant in all ranges of temperature. In the system plasma high in lactate and cells low in lactate, Q_{10} is about 2 in the range 25–36°, about 6 in the range 14–25°, and about 30 in the range 0–14°. An empirical exponential equation of the type $-\ln K = ae^{c-T} + b$ has been found to fit the data, when a, c, and b are arbitrary constants and T is the absolute temperature (Table VI). This equation illustrates the abrupt change in K as the temperature decreases.

TABLE V

Time Relations of Diffusion of Lactate from Plasma into Cells at 23°

Time after mixing	V_c	L_{WB}	L_p		L_c		$\frac{L_c}{L_p}$	
			Found	Calculated*	Found	Calculated*	Found	Calculated*
min	*ml per 100 ml*	*m eq. per l.*	*m eq. per l.*	*m.eq. per l*	*m eq. per l*	*m.eq per l*		
0.50	50	11.8	24.6	23.2	0	0.4	0	0.02
2.88		11.8	20.4	21.1	3.1	2.5	0.15	0.12
4.85		11.9	19.5	19.9	4.3	3.9	0.22	0.20
7.45	50	11.9	18.8	18.8	5.0	5.0	0.27	0.27
9.88		11.9	18.6	18.2	5.2	5.6	0.28	0.31
14.5		12.0	17.9	17.6	6.1	6.4	0.34	0.36
29.2		12.1	17.1	17.1	7.1	7.1	0.42	0.41
60.0	50	12.4	17.4	17.1	7.6	7.7	0.44	0.45

* L_p, L_c, and L_c/L_p calculated as described in the text. The equation of good fit was:

$$L_c/L_p = 0.44\left(\frac{1 - 0.76e^{-0.166t}}{1 + 0.76e^{-0.166t}}\right)$$

It will be noted that, in this particular experiment, calculation was simplified by virtue of V_c being 50.

Lactate behaves similarly to glucose and to phosphate. According to Masing's data (14), the Q_{10} for glucose is 2.3 in the range 15–25°, and 12 in the range 0.5–10°. Halpern (7) reported that inorganic phosphorus diffuses across the plasma-cell boundary rapidly at 37.5°, slowly at 23°, and not at all at 3°. She concluded that it does not obey the Gibbs-Donnan law, its behavior not being explicable by the factors involved in uncomplicated diffusion.

A combination of several factors might explain the 600-fold decrease in the "permeability constant" of lactate as the temperature drops from 38–0°. First, a normal decrease of about 125-fold would be expected for uncomplicated diffusion of substances of high Q_{10} (1). Second, there is in blood a shift of as much as 0.2 pH to the alkaline side when the temperature drops from 38–0°. Third, lowering the temperature decreases the dissociation constant of organic acids. The net result of these latter two changes might be a decrease in the concentration of undissociated lactic acid molecules, with consequent slowing down of diffusion. In their discussion of cell permeability, Davson and Danielli (1) emphasize the theoretical considerations that require a small decrease in Q_{10} with increase in temperature, and summarize thus: ". . . the permeability to an ion is defined by three sets of factors: (1) interface factors, (2) membrane 'viscosity' factor, (3) thickness factor. These factors are themselves influenced by (i) mass or radius of ion (lipoid membranes); radius of ion and pore radius (porous membranes), (ii) orientation factors, (iii) adsorption (Traube effect), (iv) values of activation energies. And these terms involve effects due to (α) diffusion potentials, (β) chemical nature of membrane, (γ) chemical nature of diffusing ion, and (δ) charge on membrane surface, and on membrane pores." The present data on lactate are not complete enough to allow analysis in terms of the factors of Davson and Danielli (1), but systematic study of such other acids as acetic, formic, and pyruvic acids by means of the present techniques might yield information of the permeability of the red cell in relation to length of carbon chain and substituent groups.

TABLE VI

Changes of "Permeability Coefficient" with Temperature

Experiment No.	Temperature	"Permeability coefficient"	
		Observed*	Calculated*
	°C.		
70	38	0.181	0.235
91	36	0.202	0.202
68	28	0.050	0.111
91	25	0.087	0.087
85	23	0.083	0.071
91	14	0.014	0.015
86	0	0.0004	0.0002
91	0	0.0003	0.0002

* The observed "permeability coefficient" for lactate is derived from experiments on various subjects. The calculated value is derived as described in the text The equation of good fit was $-\ln K = e^{0.056(310-T)} + 0.5$.

Shifts of Chloride and Bicarbonate Associated with Lactate—When lactate is added to plasma and shifts into the cells, chloride and bicarbonate move out of the cells, but relatively more chloride than bicarbonate. An experimental analysis of this shift was based on the two assumptions that when plasma high in lactate is mixed with cells that are low, samples of reconstituted blood stored at about 0° represent the initial state of the system, whereas samples warmed at 37° for 20 minutes represent the state of the system after equilibrium has been attained.

In a typical experiment blood under oil was divided into two equal portions and the plasma was removed from each. To one sample of plasma was added $l(+)$-lactic acid in 90 per cent solution, about 0.2 ml. of acid being added for every 10 ml. of plasma; a similar volume of water was added to the other sample of plasma, in order to have the same V_c in the reconstituted samples of blood. The plasma and cells were mixed at 0° and each of these samples of reconstituted blood was divided into two equal portions. One pair, representing high and low L_{WB} at the time of mixing, was kept at 0° until analysis; the other pair, representing high and low L_{WB} after equilibrium was attained, was warmed at 37° for 20 minutes with constant stirring. The conclusions to be drawn from this experiment, and others less complete (Table IV), are that mere warming of the reconstituted blood has no effects significant for the present purposes; that when lactic acid enters the plasma, chloride and bicarbonate shift into the cells and simultaneously water shifts from plasma to cells, thus equalizing osmotic pressure; and that no further adjustment in water or osmotic pressure occurs, since chloride and bicarbonate shift back to plasma as lactate enters the cells. The rates of these shifts of chloride and bicarbonate are apparently conditioned by the rate of diffusion of lactate. This is shown in the case of chloride by Table VII, which lists L_p and Cl_p at various times and temperatures, after mixing plasma high in lactate with cells that were low. It is clear that chloride leaves the cells only *pari passu* with the lactate entering them. In contrast with its behavior when associated with the movement of lactate, the rate of diffusion of chloride when not so conditioned is very rapid.

We have tried without success to measure the rate of diffusion of chloride from plasma into cells, by adding HCl to plasma, by adding NaCl to plasma, and by mixing plasma of high chloride content with cells of low content, but we have not succeeded in measuring anything but final equilibrium states even at 0°. It is apparent that the rate of diffusion of chloride along a chloride gradient is far faster than that of lactate along a lactate gradient. The work of Dirken and Mook (4), using a modification of the technique of Hartridge and Roughton (8), led to an estimate of not more than 3 seconds for chloride equilibrium to be established in whole blood, and this was confirmed for chloride and bicarbonate by Luckner and Lo-Sing (13), using electrometric methods. It is therefore not surprising that the present experimental technique does not show differences even at 0°.

DISCUSSION

The present experiments offer a possible explanation for a phenomenon observed in muscular exercise. After a sharp burst of exercise, there is a continuing increase in whole blood lactate for a few minutes after the exercise is over. This may be due not to a real production of lactate after the exercise is over, but to a slowly increasing cell lactate in a plasma that has a lactate level held essentially constant for a few minutes by the relatively large muscle mass from which lactate is passing into the extracellular fluid. Some unpublished experiments[2] have been performed on a subject who ran to exhaustion in 40 seconds. Blood was drawn and immediately cooled to 0°. The value for L_p remained essentially constant for 5 minutes, but L_c/L_p increased during this period, as would be expected from the above hypothesis.

TABLE VII

Plasma Lactate and Plasma Chloride during Diffusion of Lactate into Cells

The results are expressed in milliequivalents per liter.

Determination	Temperature	Time after mixing plasma and cells									
		0 min	1 min.	2 min	3 min	4 min.	5 min	10 min	12 min	23 min	50 min
	°C										
L_p ..	0	21.5			21.2		21.0	20.5			
Cl_p . .	0	94.2			95.2		95.1	95.8			
L_p .	25	21.5		20.3		18.7			18.3	17.3	17.4
Cl_p ..	25	94.2		95.7		96.7			97.9	98.0	98.2
L_p	36	21.5	20.2	19.4	18.8	17.3					
Cl_p....	36	94.2	96.3	96.6	97.1	97.4					

It is clear from the present work that equilibrium between red cells and plasma is sometimes reached very slowly after a change in the constituents of blood. There is good experimental justification for the convention of treating quantitative changes of the physicochemical components of the blood (9) as if they occur in stepwise fashion and not simultaneously.

SUMMARY

1. A study has been made of the physicochemical properties of blood containing lactate in concentrations representative of those seen after severe muscular exercise.

2. At equilibrium the distribution ratio of lactate between cells and plasma is of the same order of magnitude as that of chloride, and both respond in the same way to changing pCO_2 and pO_2. Therefore, both

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[2] Dill, D. B., Edwards, H. T., Talbott, J., Bock, A. V., and Daly, C., unpublished.

lactate and chloride may be treated as part of the physicochemical system by the same theoretical considerations.

3. The kinetics of the diffusion of lactate in blood are susceptible of analysis because cooling blood to 0° virtually halts the entry of lactate into the cells without altering cell volume. It is found that curves for the diffusion of lactate from plasma high in lactate into cells that are low all have the same shape and final equilibrium point at any temperature between 0–37°. This family of curves can be fitted by an application of Fick's principle. The Q_{10} of the diffusion constant is far higher at low temperatures than at high.

4. Immediately after plasma high in lactate is mixed with cells that are low in lactate, there is a shift of chloride and bicarbonate into the cells. Then as lactate moves into the cells, chloride and, to a smaller extent, bicarbonate move back into the plasma, and their movements are *pari passu* with those of lactate. When equilibrium is finally attained, which takes about 10 minutes at 37° and about 100 hours at 0°, the Gibbs-Donnan ratio for chloride is considerably below its value for blood of man at rest, and the ratio for bicarbonate is higher.

BIBLIOGRAPHY

1 Davson, H., and Danielli, J F , The permeability of natural membranes, New York (1943)
2 Dill, D B , Daly, C , and Forbes, W H , *J Biol Chem* , **117,** 569 (1937).
3 Dill, D B , Edwards, H T , and Consolazio, W. V., *J. Biol. Chem* , **118,** 635 (1937).
4 Dirken, M N. J , and Mook, H. W , *J Physiol* , **73,** 349 (1931).
5 Edwards, H T , *J Biol Chem* , **125,** 571 (1938)
6 Fick, A E , *Ann Phys u Chem* , **94,** 59 (1855)
7 Halpern, L , *J Biol Chem* , **114,** 747 (1936).
8. Hartridge, H , and Roughton, F J W , *J. Physiol* , **62,** 232 (1927)
9. Henderson, L. J , Blood, A study in general physiology, New Haven (1928)
10. Hirota, K , *J Biophysics*, Japan, **1,** 233 (1925); **2,** 283 (1927).
11. Jacobs, M H , and Stewart, D R , *J Cell. and Comp Physiol* , **1,** 71 (1932)
12. Keys, A , *J Biol Chem.*, **132,** 181 (1940).
13 Luckner, H , and Lo-Sing, T , *Arch ges Physiol* , **239,** 286 (1937)
14 Masing, E , *Arch ges Physiol* , **156,** 401 (1914)
15 Peters, J P , and Van Slyke, D. D., Quantitative clinical chemistry, Methods, Baltimore (1932).
16 Van Slyke, D D , and Sendroy, J , Jr , *J Biol. Chem* , **58,** 523 (1923–24).
17. Van Slyke, D D , Wu, H., and McLean, F. C , *J Biol Chem* , **56,** 765 (1923)

THE VITAMIN B_6 GROUP

III. THE VITAMIN ACTIVITY OF PYRIDOXAL AND PYRIDOXAMINE FOR VARIOUS ORGANISMS*

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Evidence for the production *in vivo* from pyridoxine of compounds with increased activity for certain lactic acid bacteria (1) and for the production of similar compounds by amination or oxidation procedures *in vitro* (2, 3) has been reported. Only a limited number of structures for the compounds resulting from amination or oxidation appeared possible; synthesis (4, 5) of certain of these resulted in identification of pyridoxamine and pyridoxal as the active compounds (6).

In the present paper, the activity of these compounds in promoting growth of a number of different organisms is compared with that of pyridoxine. A brief summary of such results with three microorganisms has previously appeared (6).

EXPERIMENTAL

A number of different organisms[1] which have been reported to require added pyridoxine for growth were used. Assays for growth-promoting activity of the various compounds were carried out by published procedures, or by slight modifications of them, as noted in each individual case below.

Streptococcus faecalis R (*Streptococcus lactis R*)—This organism was that originally used in the discovery of "pseudopyridoxine" (1), and was used together with *Lactobacillus casei* in implicating an amine and an aldehyde as structures for pyridoxamine and pyridoxal (3). As previously noted (3), trouble is occasionally encountered in applying the assay with this organism under conditions as originally described. Others have encountered similar difficulties (7). While the reasons for such erratic behavior are not entirely clear, the defect appeared to lie in the presence of growth-promoting substances in the hydrolyzed casein. The following modified method of preparation gave a much superior product for the purpose at hand.

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* Presented in part before the 108th meeting of the American Chemical Society at New York, September 11-15, 1944

[1] We are indebted to the following individuals who kindly supplied the cultures indicated: Dr. C. F. Niven (*Streptococcus lactis* L101, *Streptococcus mastitidis* G-2, *Streptococcus mastitidis* 97B), Dr. E. McCoy (*Streptococcus zymogenes* H69D5 and *Bacillus lactis acidi* Bl-1), Dr. G. W. Beadle (*Neurospora sitophila* 299), Dr. W. J. Robbins (*Ceratostomella ulmi*), Dr. V. G. Lilly (*Mycoderma valida*), Dr. P. R. Burkholder (*Saccharomyces oviformis*), and Dr. C. N. Frey (*Saccharomyces carlsbergensis*)

50 gm. of Labco vitamin-free casein were refluxed for 8 to 10 hours with 500 cc. of constant boiling HCl. Excess HCl was removed by concentration to a heavy syrup under reduced pressure. The syrup was dissolved in water, and the concentration repeated. The product was dissolved, adjusted to pH 7.0 with concentrated NaOH solution, and diluted to 500 cc. This solution was stirred for 15 minutes with 5 gm. of activated charcoal (Darco G-60) and filtered. The charcoal treatment was repeated, and the resulting solution stored under toluene until used.

TABLE I

Composition of Basal Medium

	Amount per 100 cc of medium (20 assay tubes)
	mg
Hydrolyzed casein (vitamin-free, charcoal-treated)	1000
Sodium acetate	1200
Glucose	2000
Asparagine	20.0
Tryptophane	10.0
Cystine hydrochloride	20.0
Adenine sulfate	2 0
Guanine hydrochloride	2.0
Uracil	2 0
	γ
Thiamine chloride	20
Calcium pantothenate	40
Riboflavin	40
Nicotinic acid	40
Folic acid concentrate* ..	6.6
Biotin (free acid)	0.08
p-Aminobenzoic acid	20.0
	cc
Inorganic Salts A†	1.0
" " B†	1.0

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* The folic acid concentrate used had a "potency" of 3000, and was prepared by carrying the procedure for concentration of folic acid (8) through adsorption and elution from Lloyd's reagent Preparations of much less purity can be used successfully if added in correspondingly greater amount; the amount added is about 5 times that necessary for maximum growth.

† Snell and Strong (9).

The composition of the basal medium is shown in Table I. This is twice the concentration used in the final assay tubes. For use with *Streptococcus faecalis*, 25 mg. of K_2HPO_4, 25 mg. of KH_2PO_4, and 2 mg. of glycine were added per assay tube.[2] The medium was adjusted to pH 6.8 for use.

The same medium was used for strains of *Streptococcus lactis*. *Streptococcus zymogenes* and *Streptococcus mastitidis* G-2 and 97B were also cultured in this medium, but the initial pH was adjusted to 7.4 rather than pH 6.8.

For *Lactobacillus casei* and *Bacillus lactis acidi*, the basal medium shown in Table I was modified by addition of 2 mg. of *dl*-alanine (13) per tube. For *Lactobacillus arabinosus*, the unmodified basal medium was used. The initial pH of the medium was 6.8 with each of these three organisms. With all these bacteria, assays were carried out in the same manner as previously described with modifications noted below (1). The incubation time and temperature are given with the tables. Growth responses were determined turbidimetrically. In all cases, inocula were grown in the medium used for assay, but supplemented with pyridoxine. The organisms were centrifuged out after 16 to 24 hours growth, resuspended in 0.9 per cent saline, and 1 drop of a suspension of barely visible turbidity used in inoculating each tube.

The pyridoxine-requiring, mutant strain of *Neurospora sitophila* (14) was used for assay in accordance with the directions of Stokes *et al.* (15). Assays with *Ceratostomella ulmi* were carried out as described by Robbins and Ma (16). Determinations with *Mycoderma valida* were made by an unpublished method of Lilly and Leonian.[3]

Assays were also made with three species of yeast according to published procedures. These were *Saccharomyces carlsbergensis* (17), *Saccharomyces cerevisiae* (18), and *Saccharomyces oviformis* (19). Tests with *Saccharomyces carlsbergensis* and *Saccharomyces oviformis* were carried out on one-fifth the scale recommended by authors of these methods; the assay tubes were diluted with 5 cc. of saturated aqueous chlorothymol solution preliminary to determinations of turbidity.

Assays with rats were made by the method of Conger and Elvehjem (20).

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[2] Luckey *et al.* (10) showed that additional potassium salts greatly improved growth of *Streptococcus faecalis* on a very similar medium used for folic acid assay (11); the same effect is noted in the above medium Addition of glycine insures low "blank" tubes. It prevents response of this organism to alanine and traces of other substances which have vitamin B_6 activity for this organism. This growth-depressing effect of glycine is completely and specifically overcome by substances having vitamin B_6 activity for this organism (12).

[3] We wish to thank Dr. Lilly for furnishing details of this method in advance of publication.

Results

Streptococcus faecalis R—Detailed results comparing the activity of pyridoxine, pyridoxamine, and pyridoxal for this organism are given in Table II. Sterile solutions containing the amounts of material indicated were added to the medium after the assay tubes had been sterilized and cooled. This was done to prevent formation during autoclaving of substances with increased activity from pyridoxine (2). Pyridoxamine was about 8000 times, pyridoxal about 5500 times, as active in promoting growth as was pyridoxine hydrochloride. It was previously shown (1) that no detectable amount of pyridoxine was absorbed from the medium during growth of this organism. These high figures for the activities of pyridoxamine and pyridoxal do not, therefore, mean that 8000 γ of pyridoxine are required in the organism to function as does only 1 γ of pyridoxamine. They simply express the low yield in which pyridoxine is converted to substances having this high degree of activity. Whether this conversion can be effected to any degree by *Streptococcus faecalis* itself, or whether it occurs only through interaction of pyridoxine with constituents of the assay medium, is an unsettled point, but available evidence favors the latter explanation. Several assays of this nature have

TABLE II

*Comparison of Growth-Promoting Effect of Pyridoxine, Pyridoxal, and Pyridoxamine for Streptococcus faecalis R**

Compound added	Amount	Galvanometer reading	Pyridoxine equivalent		Average pyridoxine equivalent
	γ per 10 cc		*γ*	*γ per γ compound*	
Pyridoxine hydrochloride	0.0	10			
	1.0	11			
	2.0	15			
	4.0	28			
	8.0	52			
	16.0	70			1.0
Pyridoxamine	0.0005	28	4.0	8000	
	0.001	56	8.8	8800	
	0.002	69	14.5	7250	
	0.003	77			8000
Pyridoxal	0.0005	20	2.8	5600	
	0.001	37	5.4	5400	
	0.002	63	11.0	5500	
	0.004	77			5500

* Incubation time, 16 hours at 30° A galvanometer reading of 0 was given by distilled water, a reading of 100 was given by an opaque object

yielded values for the activity of pyridoxamine which varied from 500 to 12,000 times that of pyridoxine hydrochloride in the same test. The activity of pyridoxal similarly varied from 400 to about 8000 times that of pyridoxine. It should be emphasized that the variation observed is due chiefly to variation in the apparent activity of pyridoxine; the amount of pyridoxamine or pyridoxal required for maximum or half maximum growth is relatively constant. Thus it would be more accurate to ascribe an activity of 1.0 to pyridoxamine, when pyridoxal would have an activity of about 0.7 and pyridoxine hydrochloride about 0.00008 to 0.002.

Lactobacillus casei—The activities of pyridoxine hydrochloride, pyridoxamine, and pyridoxal were determined as described above, except that *L. casei* was used as the test organism. Again, sterilization of samples with the medium was avoided to prevent changes in activity due to interaction of these compounds with constituents of the medium. The results are given in Table III. Pyridoxamine was about 10 times as active, pyridoxal about 1400 times as active, as pyridoxine hydrochloride on an equal weight basis. Repeated assays have yielded values for the activity of pyridoxamine which ranged from 3 to 30 times that of pyridoxine hydrochloride. Pyridoxal, similarly tested, was 1000 to 1500 times as active as pyridoxine. Separate experiments similar to those reported before with *Streptococcus faecalis* (1) showed that neither pyridoxine nor pyridoxamine was absorbed from the medium to any appreciable extent by *L. casei;* presumably, as in the case of *Streptococcus faecalis*, derived products, formed in very small amount, are the substances absorbed and utilized. Pyridoxal was completely removed from the medium during growth, so far as could be determined.

TABLE III

*Comparative Activity of Pyridoxine, Pyridoxamine, and Pyridoxal for Lactobacillus casei**

Compound	Active range	Amount producing half maximum turbidimeter reading	Comparative activity
	γ per 10 cc	*γ per 10 cc*	
Pyridoxine hydrochloride	0.2 -10	2.0	1.0
Pyridoxamine	0 02 - 1.0	0.2	10.0
Pyridoxal	0 0003- 0 01	0 0014	1430.0

* Incubated 24 hours at 37°.

Saccharomyces carlsbergensis—A summary of the activity of pyridoxine, pyridoxamine, and pyridoxal for this organism is given in Table IV. If all compounds had equal activity on a molecular basis, pyridoxamine and pyridoxal should be 1.22 times as active as pyridoxine hydrochloride. It is evident that this ratio is closely approached. In repeated tests, activity of pyridoxamine varied from 0.8 to 1.3 times that of pyridoxine hydrochloride, while that of pyridoxal varied from 0.9 to 1.4 times that of pyridoxine hydrochloride.[4]

Effect of Autoclaving with Casein—In each of the above tests, sterile solutions of the sample to be tested were added to the medium in the assay tubes after these had been sterilized and cooled. This was done because autoclaving with the medium is known to alter the activity of pyridoxine for *Streptococcus faecalis* and *Lactobacillus casei* (2). It appeared possible that autoclaving pyridoxamine or pyridoxal with the medium might alter their activity. This proved to be the case. Pyridoxal (10 γ) was added to 1 cc. of a solution containing casein hydrolysate equivalent to 100 mg. of casein. Similar tubes containing 10 γ of pyridoxamine and 1.0 mg. of pyridoxine hydrochloride were prepared. The casein hydrolysate was that used in preparation of the basal medium, and was at pH 7.0. All tubes were autoclaved at 15 pounds steam pressure for 30 minutes, cooled, and diluted for assay. Similar samples, unheated, served as controls. The results are given in Table V. The activity of pyridoxine for *S. faecalis* is greatly increased by this procedure, as previously reported. Its activity for *L. casei* is increased to a lesser extent, while for yeast its activity remains essentially unchanged. The activity of pyridoxamine for these three organisms is unchanged by the treatment. With pyridoxal, no change in activity occurs for yeast. With *S. faecalis*, however, autoclaving with casein increases the activity of this compound to that of pyridoxamine as a limit. With *L. casei*, activity of the aldehyde is greatly decreased by autoclaving with casein. Separate experiments, with longer autoclaving times, showed that this decrease proceeds until the compound has approximately the same activity as pyridoxamine for *L. casei*. This behavior

TABLE IV

Comparative Activity of Pyridoxine Hydrochloride, Pyridoxamine, and Pyridoxal for Saccharomyces carlsbergensis

Compound with active range of 0 002-0 04 γ per 10 cc	Amount producing half maximum turbidimeter reading	Comparative activity
	γ per 10 cc	
Pyridoxine hydrochloride	0.008	1.0
Pyridoxamine	0 0060	1 33
Pyridoxal	0 0056	1 42

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[4] In some cases, lower values (0.25 to 0.75) were obtained for the activity of pyridoxamine; these were traced to partial inactivation of the compound which occurred when it was autoclaved in very dilute aqueous solutions

with all three organisms suggests that pyridoxal may be converted to pyridoxamine by this treatment. This could occur readily by transamination reactions (21). An alternate explanation would be formation of stable intermediate compounds, such as Schiff's bases or compounds derived from condensation of amino acids with the cyclic form of pyridoxal (4, 5), possessing activity equivalent to that of pyridoxamine.

Other Microorganisms—The comparative effectiveness of these three compounds for several additional microorganisms is summarized in Table VI. The shape of the growth curve produced by increasing concentrations of various compounds differed somewhat with some of the bacteria, so that figures obtained for the comparative activity of the compounds at successive concentration levels drifted considerably in some cases. For this reason it is dangerous to draw elaborate conclusions. Some general conclusions, however, are unmistakable. For all bacteria tested, both pyridoxamine and pyridoxal are much more effective in promoting growth than is pyridoxine. Almost without exception, the activity of pyridoxal is decreased by autoclaving with the medium, in some cases, the potency of pyridoxamine appears to be increased by such treatment. These changes in activity are apparently produced by interaction of these compounds with the various components of the medium. Such interaction may well prove to be important to fulfilment of the biochemical function of pyridoxal and pyridoxamine.

TABLE V

Effect of Autoclaving with Casein Hydrolysate on Activity of Pyridoxine, Pyridoxamine, and Pyridoxal

Compound		Comparative activity		
		Saccharomyces carlsbergensis	*Streptococcus faecalis*	*Lactobacillus casei*
Pyridoxine hydrochloride	Untreated	1.0	1.0	1.0
	Autoclaved with casein	0.9	48.0	2.7
Pyridoxamine	Untreated	1.23	8700	6.0
	Autoclaved with casein	1.30	8700	7.1
Pyridoxal	Untreated	1.25	4400	1150
	Autoclaved with casein	1.34	8500	69

It has been shown previously that for one yeast, *Saccharomyces carlsbergensis*, an organism well suited for the microbiological determination of pyridoxine (17), pyridoxamine and pyridoxal approximately equal pyridoxine in activity when suitable precautions are taken to prevent changes in these compounds preliminary to assay. Repeated assays with *Sac-*

charomyces cerevisiae GM and *Saccharomyces oviformis* gave lower values for the potency of these two compounds, as shown in Table VI. Their apparent potency was lowered still further by autoclaving or steaming with water which contained air. Separate tests showed that with both organisms the comparative activity increased as the growth period was lengthened (Table VII). Thus the rate at which these compounds are utilized appears to be somewhat lower than that at which pyridoxine is utilized by these organisms.

TABLE VI

Activity of Pyridoxine, Pyridoxamine, and Pyridoxal for Miscellaneous Microorganisms

Organism	Average comparative activity					
	Unautoclaved*			Autoclaved†		
	Pyridoxine hydrochloride‡	Pyridoxamine	Pyridoxal	Pyridoxine hydrochloride‡	Pyridoxamine	Pyridoxal
Bacteria§						
Streptococcus lactis L101.	1.0 (3 γ)	330	1200	1.0 (1 γ)	250	160
“ “ UT	1.0 (30 “)	930	580	1.0 (5 “)	640	160
“ “ 374	1.0 (10 “)	300	760	1.0 (3 “)	210	96
“ *mastitidis* G-2	1.0 (10 “)	31	650	1.0 (3 “)	250	140
Streptococcus mastitidis 97B	1.0 (10 “)	190	760	1.0 (10 “)	370	130
Streptococcus zymogenes H69D5	1.0 (20 “)	350	400	1.0 (20 “)	570	170
Bacillus lactis acidi Bl-1	1.0 (3 “)	19	480	1.0 (2 “)	7	68
Yeasts						
Saccharomyces oviformis	1.0	0.22–0.75	0.63–1.2			
“ *cerevisiae* GM	1.0	0.16–0.64	0.41–1.2			
Molds						
Ceratostomella ulmi	1.0	2.4	1.2			
Mycoderma valida	1.0	1.4	1.5			
Neurospora sitophila 299	1.0	1.4	1.4			

* The media were sterilized by autoclaving before addition of the samples; after cooling, sterile solutions containing the substances to be tested were added aseptically.

† The samples were added to the medium as usual, and the assays autoclaved for 15 minutes at 15 pounds pressure.

‡ Pyridoxine hydrochloride is used as a standard with a potency of 1.0 in both the "unautoclaved" and "autoclaved" tests. Its own growth-promoting power is often changed by autoclaving, however. The figures in parentheses give the micrograms per 10 cc. which produced near maximum growth under the test conditions.

§ All bacterial cultures were incubated at 37° for 24 hours.

For the molds tested, pyridoxamine and pyridoxal are equally or slightly more active than pyridoxine on a molar basis. Mycelial development of *Ceratostomella ulmi* occurred more rapidly with pyridoxamine than with pyridoxine or pyridoxal. Robbins and Ma (16) recommend a 9 to 20 day incubation period for this growth test; the former was used in the present work. It is quite possible that on longer incubation more nearly equal figures for the activity of the three compounds would be obtained.

TABLE VII

*Effect of Increased Time of Incubation on Relative Activity of Pyridoxal and Pyridoxamine for Yeast**

Time of incubation	Relative potency (pyridoxine hydrochloride = 1.0)			
	Saccharomyces oviformis		*Saccharomyces cerevisiae*	
	Pyridoxal	Pyridoxamine	Pyridoxal	Pyridoxamine
hrs				
16	0.63	0.22	0.41	0.16
21	0.71	0.33	0.58	0.26
27	0.83	0.40	1.20	0.40

* The samples were steamed separately in oxygen-free water before being added to the tests, and were not heated with the medium

TABLE VIII

*Comparative Activity of Pyridoxine, Pyridoxal, and Pyridoxamine in Accelerating Growth of Lactobacillus arabinosus**

Pyridoxine hydrochloride		Pyridoxamine		Pyridoxal	
Amount added	Galvanometer reading	Amount added	Galvanometer reading	Amount added	Galvanometer reading
γ per 10 cc		*γ per 10 cc*		*γ per 10 cc.*	
0	66	0	66	0	66
3	71	0.0005	82	0.0005	70
10	75	0.001	87	0.001	73
30	80	0.003	91	0.003	76
100	84	0.01	92	0.01	82
300	90	0.03	92	0.03	88

* All tubes were incubated at 30° for 18 hours, chilled in an ice box to stop growth, and read turbidimetrically. A reading of 0 is given by distilled water; a reading of 100 represents no light transmitted

Lactobacillus arabinosus—This organism is representative of a number of lactic acid bacteria which do not require added pyridoxine for growth (22). Addition of pyridoxine increases the rate of growth over limited periods of time, however (23). In view of the comparative inactivity of pyridoxine in promoting growth of other species of lactic acid bacteria, it appeared possible that the rate at which pyridoxine was converted to the form of this vitamin which is actually utilized might limit growth rate of the organism. The activity of these compounds in promoting early growth of *Lactobacillus arabinosus* is shown in Table VIII. None of the samples was heated with the medium. Pyridoxamine is more than 100,000 times as effective as pyridoxine hydrochloride in promoting growth of this organism; pyridoxal is about 5000 times as active. It is obvious that these substances, especially pyridoxamine, are much more readily available to the organism than is pyridoxine. Separate experiments showed that for this organism, as for *Streptococcus faecalis*, sterilization with the medium greatly increased the apparent activity of pyridoxine. The activity of pyridoxamine was unchanged by this procedure, while that of pyridoxal was likewise increased.

TABLE IX

Comparative Activity of Pyridoxine and Related Compounds for White Rats

Compound	Amount fed	Average gain per animal*	Comparative activity
	γ per day	*gm. per wk*	
Pyridoxine hydrochloride	0	3.5	1.0
	2	10.2	
	4	13.2	
	6	14.6	
	10	18.4	
Pyridoxamine	4	15.6	1 6
Pyridoxal	4	14.0	1.2
2-Methyl-3-hydroxy-4-hydroxymethyl-5-aminomethylpyridine dihydrochloride	500	10.5	0.0044

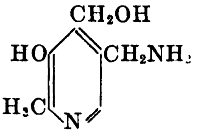
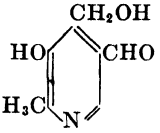
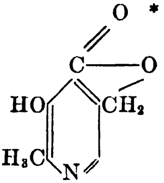
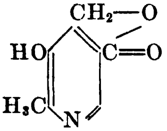
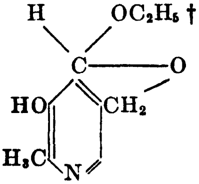
* Five animals were used for each point. Supplementation was continued for 5 weeks.

White Rats—The results of an assay with rats are shown in Table IX. Pyridoxamine and pyridoxal equal pyridoxine in activity for these animals. Whether the slightly increased activity of pyridoxamine over that of pyridoxine is real or within the experimental error of the assay cannot be said at present. The isomer of pyridoxamine containing the aminomethyl group in the 5 rather than the 4 position has only negligible activity. Thus the activity of these compounds for rats checks very closely their relative activities for *Saccharomyces carlsbergensis* and *Neurospora sitophila.*

Specificity Relationships—Data concerning the activity of previously available products related to pyridoxine have been presented (3). In Table X are summarized data concerning other products synthesized in the

TABLE X

Activity of Compounds Related to Pyridoxamine and Pyridoxal for Various Organisms

Compound	Activity for		
	Streptococcus faecalis R	*Lactobacillus casei*	*Saccharomyces carlsbergensis*
Pyridoxamine	1.0	0 007	1.0
Pyridoxal	0.4 -0.7	1.0	1.0
Pyridoxine	0 0001-0 001	0 00066-0.001	1.0
	0 00002	0.00005	0.0022
	0.00004	0.00026	0.29-0.73
 *	0.000053 (*Ca*)	0 0001 (*Ca*)	0.00025
	Inactive	Inactive	Inactive
 †	0.24	0.73	1 0

* Kindly supplied by Dr. Perlzweig The free acid from which the lactone was prepared (4-pyridoxic acid (24)) had less than 0 01 the activity of the lactone for all three organisms. We are indebted to Dr. Karl Folkers for all other compounds.

† This compound is very easily hydrolyzed to pyridoxal (5). If autoclaved with water before testing, it shows full activity for all organisms.

course of this investigation (5). The activity of any product for an organism is given in terms of the most active substance for that organism. It is evident that the response of these organisms to pyridoxamine and pyridoxal is specific. The isomeric amine and aldehyde are less active than pyridoxine for all organisms. For yeast, the 5-formyl derivative has activity approaching that of pyridoxine, indicating that this organism is able to change it readily into the proper form for use. The inactivity of 4-pyridoxic acid and its lactone is especially interesting, since this product is very closely related to pyridoxal, and has been isolated from urine as a degradation product of pyridoxine (24).

DISCUSSION

In originally predicting possible structures for pyridoxamine and pyridoxal, the assumptions were made that similar biochemical rôles were being fulfilled by all substances which showed vitamin B_6 activity for any organism, and that such substances showed activity only after being converted to a common necessary catalyst within the organism (3, 6). It was difficult to see how, if pyridoxine itself were this catalyst, some other substance could be several hundred times as active for any organism as was pyridoxine. Consequently, it was thought that substances which showed such increased activity must be more closely related to the functional catalyst than was pyridoxine, or at least more easily transformed into this catalyst by these test organisms. It was therefore expected that these compounds would prove to have activity for all other organisms for which pyridoxine was active; and that this activity, though it might be indefinitely greater than that of pyridoxine, would probably not be markedly less. In general, this expectation is well fulfilled. Pyridoxamine and pyridoxal were as active, or more active, than pyridoxine for fifteen of seventeen organisms tested. These included molds, yeasts, lactic acid bacteria, hemolytic streptococci, and rats. For two species of yeast, pyridoxamine, though active, was somewhat less active than pyridoxine. For the same two organisms, pyridoxal sometimes showed activity equal to that of pyridoxine, but occasionally lower. The activity of these compounds increased relative to that of pyridoxine as the incubation time was increased, indicating that the three substances differed in rate of utilization, rather than in intrinsic activity. Such slight differences in activity may be due to differences in absorption rate, or other factors than structural relationship to a functional catalyst. If pyridoxamine occurs naturally, it is quite possible that the low values obtained for pyridoxine when *Saccharomyces cerevisiae* is used for assay (18) are partially due to limited availability of this substance to this organism. Lactic acid bacteria, on the other hand, if they utilize pyridoxine at all, do so at a vanishingly small rate, since growth ceases before maximum values are reached in the presence of excess pyridoxine (2, 25).

Scudi (26) and Harris (27) have pointed out the ease with which various reactions involving the 4-hydroxymethyl group of pyridoxine occur; and there is little reason to suspect that the 4-aminomethyl group would confer added stability. Condensations between compounds containing carbonyl groups and amino acids on heating are well known. It is therefore not surprising that the autoclaving of these three compounds with the medium or with amino acids profoundly affects their activity as subsequently determined by certain microorganisms. The effect of such treatment on the activity of a compound depends upon the test organism used. Several cases have been observed above. (*a*) The activity of pyridoxine is greatly increased for *Streptococcus faecalis*, and somewhat increased for *Lactobacillus casei*, but is essentially unchanged for *Saccharomyces carlsbergensis;* (*b*) activity of pyridoxal is increased by autoclaving with amino acids for *S. faecalis*, *Lactobacillus arabinosus*, and certain other organisms, is greatly decreased for *Lactobacillus casei*, and remains essentially unchanged for *S. carlsbergensis;* and (*c*) activity of pyridoxamine remains unchanged for *S. carlsbergensis*, *S. faecalis*, and *Lactobacillus arabinosus*, but may be increased for organisms such as *Streptococcus mastitidis.* Autoclaving in very dilute aqueous solutions containing dissolved air lowers the activity of these compounds. Unpublished data show that like pyridoxine (28), these compounds are readily inactivated by light, especially when in dilute solution.

In an early study of the pyridoxine nutrition of *Lactobacillus casei*, Bohonos *et al.* (29) showed that to promote equal levels of growth much higher concentrations of pyridoxine were required when strictly anaerobic conditions were maintained, and that with increasing oxygen tension smaller amounts of pyridoxine were required. This result is now readily explicable: pyridoxine itself does not promote growth of this organism, its partial oxidation product does, and formation of the latter is favored by oxidizing conditions.

When pyridoxamine or pyridoxal is used as standard with *Streptococcus faecalis*, values for the "vitamin B_6" activity of natural materials are obtained which are as low or lower than those indicated by yeast assay. Similarly, if pyridoxal is used as the standard with *Lactobacillus casei*, values for the "vitamin B_6" content of tissues are obtained which are considerably lower than those obtained by yeast or *Neurospora* assay. Such values stand in marked contrast to the absurdly high values obtained against a pyridoxine standard, which first led to an investigation of this problem (1). Considerable evidence indicates that pyridoxamine and pyridoxal, or higher combinations of these compounds, are chiefly responsible for the "pseudopyridoxine" activity of natural materials. This evidence will be presented separately (30). If this is true, it indicates that "pseudopyridoxine" has activity for all organisms, including animals, but that this activity, for rats at least, is about equal to that of pyridoxine. It follows that for determinations of total "vitamin B_6" activity organisms for which these various forms have approximately equal activity should be used. This requirement is met by rats, *Saccharomyces carlsbergensis*, and *Neurospora sitophila*. The latter two microorganisms have already been recommended for use in assay work (17, 15).

SUMMARY

Pyridoxamine, pyridoxal, and pyridoxine were compared with respect to their growth-promoting activity for seventeen different organisms. For one group, represented by some yeasts, molds, and rats, these compounds have approximately equal activities on a molar basis. For two yeasts, pyridoxamine and pyridoxal showed variable activity, which was usually, but not always, somewhat less than that of pyridoxine. For a large group of lactic acid bacteria the activity of pyridoxamine and pyridoxal varies from a few fold to several thousand-fold that of pyridoxine; it is doubtful whether these organisms utilize unchanged pyridoxine at all.

Pyridoxal and pyridoxamine are labile compounds which readily undergo reaction with various constituents of the basal media when heated. Such reaction can be detected by the change in growth-promoting activity produced by autoclaving with the medium. The magnitude and direction of this change vary with different test organisms. The compounds are also labile to heating in dilute water solutions containing dissolved air, and are destroyed by light.

Various related structures were tested for activity, and the results reported. The response of lactic acid bacteria to pyridoxal and pyridoxamine is very specific.

Implications of some of the results are discussed.

Addendum—Since submission of this paper, rat assays have been concluded on the lactone of 4-pyridoxic acid, and upon 2-methyl-3-hydroxy-4-hydroxymethyl-5-formylpyridine (*cf* Table X) The lactone did not promote growth at levels of 4 γ or of 400 γ per rat per day The aldehyde was inactive at a level of 4 γ per rat per day Pyridoxal, used as the standard, gave an average growth rate of 12 7 gm. per week per rat at a level of 2 γ per rat per day. The control group (no vitamin B_6) grew at an average rate of 2 4 gm. per week per rat. Four animals were used per group, and supplements were fed for 7 weeks.

BIBLIOGRAPHY

1. Snell, E. E., Guirard, B. M., and Williams, R. J., *J. Biol. Chem.*, **143**, 519 (1942).
2. Snell, E. E., *Proc Soc. Exp. Biol. and Med.*, **51**, 356 (1942).
3. Snell, E. E., *J. Am. Chem. Soc.*, in press.

4. Harris, S. A., Heyl, D., and Folkers, K., *J. Biol. Chem.*, **154,** 315 (1944).
5. Harris, S. A , Heyl, D , and Folkers, K., *J. Am. Chem. Soc* , in press.
6. Snell, E. E., *J. Biol. Chem.*, **154,** 313 (1944).
7. Carpenter, L. E., and Strong, F. M., *Arch Biochem.*, **3,** 375 (1944).
8. Mitchell, H. K., Snell, E. E , and Williams, R. J., *J. Am. Chem. Soc.*, **66,** 267 (1944).
9. Snell, E. E., and Strong, F. M , *Ind. and Eng. Chem., Anal. Ed.*, **11,** 346 (1939).
10. Luckey, T. D., Briggs, G M., Jr , and Elvehjem, C A., *J. Biol Chem.*, **152,** 157 (1944)
11. Mitchell, H K , and Snell, E E , *Univ. Texas Pub., No. 4137*, 36 (1941)
12. Snell, E E., and Guirard, B. M , *Proc. Nat. Acad. Sc.*, **29,** 66 (1943).
13. Snell, E E., *Proc. Soc. Exp. Biol. and Med.*, **55,** 36 (1944)
14. Beadle, G W , and Tatum, E. L , *Proc. Nat. Acad. Sc.*, **27,** 499 (1941).
15 Stokes, J. L., Larsen, A , Woodward, C R , Jr , and Foster, J. W , *J. Biol. Chem.*, **150,** 17 (1943).
16. Robbins, W. J., and Ma, R , *Proc Nat Acad Sc.*, **29,** 172 (1943).
17. Atkin, L , Schultz, A. S , Williams, W. L , and Frey, C. N., *Ind. and Eng Chem., Anal. Ed.*, **15,** 141 (1943).
18. Williams, R. J., Eakin, R. E , and McMahan, J. R., *Univ. Texas Pub , No. 4137*, 24 (1941).
19. Burkholder, P. R , *Am J. Bot* , **30,** 206 (1943)
20 Conger, T. W , and Elvehjem, C. A , *J Biol Chem* , **138,** 555 (1941)
21 Herbst, M , and Rittenberg, D , *J. Org Chem.*, **8,** 380 (1943)
22. Bohonos, N., Hutchings, B L , and Peterson, W H , *J. Bact* , **41,** 40 (1941).
23 Snell, E E , and Wright, L D , *J Biol Chem* , **139,** 675 (1941).
24 Huff, J W , and Perlzweig, W A , *J. Biol Chem* , **155,** 345 (1944)
25. Johnson, B C , *Proc Soc Exp Biol and Med* , **55,** 199 (1944)
26 Scudi, J V., *J Biol Chem* , **139,** 707 (1941)
27. Harris, S A., *J. Am Chem Soc* , **62,** 3203 (1940), **63,** 3363 (1941)
28. Hochberg, M , Melnick, D , Siegel, L , and Oser, B. L , *J. Biol Chem* , **148,** 253 (1943).
29. Bohonos, N , Hutchings, B L , and Peterson, W H , *J. Bact* , **44,** 479 (1942).
30 Snell, E E., *J. Biol Chem* , **157,** 491 (1945).

THE VITAMIN B_6 GROUP

IV. EVIDENCE FOR THE OCCURRENCE OF PYRIDOXAMINE AND PYRIDOXAL IN NATURAL PRODUCTS*

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In a previous paper (1) evidence for the occurrence in natural products of one or more substances which far surpassed pyridoxine in growth-promoting activity for various species of lactic acid bacteria was described. Pyridoxine was partially transformed into this substance by the animal organism. The substance responsible for the increased activity was tentatively called "pseudopyridoxine." Subsequently, it was found that substances of similarly high activity for these organisms were formed when pyridoxine was subjected to procedures causing amination or partial oxidation (2–4). The active substances formed by these procedures were identified and named pyridoxamine and pyridoxal, respectively (4–6).

The comparative activity of pyridoxamine, pyridoxine, and pyridoxal in promoting growth of a number of organisms has been described (4, 6). Among these organisms were three which behaved very differently toward the three compounds. For *Saccharomyces carlsbergensis* the three compounds were approximately equally active on the molar basis. For *Streptococcus faecalis* R, pyridoxamine was highly active, pyridoxal somewhat less active, and pyridoxine practically inactive. For *Lactobacillus casei* pyridoxal was highly active, pyridoxamine and pyridoxine were comparatively only very slightly active. Unless the latter two compounds were present in very high concentration relative to the pyridoxal concentration, activity due to them could be safely neglected without introducing significant error into the determination of pyridoxal.

By using all three organisms with proper standards, it is thus possible, by solving a very simple set of simultaneous equations, to determine the amount of any one of these compounds present in a mixture of the three. Application of the differential assay to natural materials would, of course, result in classification of the growth-promoting substances present into three fractions, depending upon whether they behaved toward the test organisms as pyridoxine, pyridoxamine, or pyridoxal. Treatment of the extracts by procedures designed to inactivate pyridoxamine or pyridoxal, followed by reassay, would permit a decision as to whether the active substances present in any fraction behaved in the predicted manner. If so, it would strongly indicate that these compounds actually occurred in nature.

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Such experients are described in the present paper. Results obtained indicate that pyridoxamine and pyridoxal occur naturally, and that a considerable portion of the "vitamin B_6" activity of natural materials is due to their presence. Their presence similarly explains satisfactorily the "pseudopyridoxine" activity of natural materials, as originally described (1).

EXPERIMENTAL

Testing Methods—Assays with *Saccharomyces carlsbergensis* were made by the method of Atkin *et al.* (7), except that they were carried out on one-fifth the scale recommended (7). Tests with *Streptococcus faecalis* R and *Lactobacillus casei* were performed as described in Paper III of this series. In all cases, samples for assay were added aseptically to previously sterilized media to prevent variable changes in activity which occur when the various compounds are autoclaved with test media (6).

Repeated tests on the same samples have shown that in their present state of development assays with either *Streptococcus faecalis* or *Lactobacillus casei* yield results which vary somewhat from time to time when applied to the same natural extracts. For this reason, all experiments described below have been run repeatedly. All figures given are those obtained on a single assay run, and are strictly comparable. While the absolute magnitude of the figures obtained on repetition of the experiment may vary slightly, the changes in activity, from which conclusions are drawn, are always present.

Differential Assay of Natural Materials—Yeast extract (Bacto), liver extract (Wilson's 1:20), rice bran concentrate (vitab), and powdered dehydrated grass juice (Cerophyl Laboratories) were used as representative of rather widely divergent types of materials. 2 per cent solutions of these were hydrolyzed with 2 N H_2SO_4, as described by Siegel *et al.* (8). Unhydrolyzed samples and neutralized, hydrolyzed samples were then assayed with the three test organisms. Pyridoxine hydrochloride was used as the standard with *Saccharomyces carlsbergensis* (the results are recalculated in terms of the free base); pyridoxamine was used as the standard for *Streptococcus faecalis* and pyridoxal for *Lactobacillus casei*. The results are given in Table I.

It is apparent that the total assay figures for the extracts obtained with each organism are generally increased by acid hydrolysis. When pyridoxamine is used as the standard for *Streptococcus faecalis*, and pyridoxal for *Lactobacillus casei*, the assay figures obtained are of the same order of magnitude as those obtained by yeast assay. These contrast with the absurdly high values obtained against a pyridoxine standard.[1] If the active substances which occur naturally promote growth of all three test organisms, and if no substances occur naturally which are more active than pyridoxamine or pyridoxal for *Streptococcus lactis* or *Lactobacillus casei*, the assays obtained by means of the latter organisms should be no higher than those obtained with yeast against a pyridoxine standard. This is true in every case except with liver extract, in which assay with *Streptococcus faecalis* against a pyridoxamine standard yields a higher result than is indicated by yeast assay. This result may be due to non-specific stimulation by substances which improve the growth of the test organism on the assay medium, or it may indicate the presence of active materials other than pyridoxamine and pyridoxal.

TABLE I

Assay of Active Substances and Their Extraction from Natural Materials

Substance	*Saccharomyces carlsbergensis* (pyridoxine standard)		*Streptococcus faecalis* (pyridoxamine standard)		*Lactobacillus casei* (pyridoxal standard)	
	Untreated	Acid-hydrolyzed	Untreated	Acid-hydrolyzed	Untreated	Acid-hydrolyzed
	γ per γ	*γ per γ*	*γ per γ*	*γ per γ*	*γ per γ*	*γ per γ*
Pyridoxine	1.00	1.00	0.0005	0.0005	0.0008	0.0008
Pyridoxamine	1.20	1.20	1.00	1.00	0.0023	0.0023
Pyridoxal.. .	1.24	1.24	0.37	0.36	1.00	1.00
	γ per gm	*γ per gm*	*γ per gm*	*γ per gm*	*γ per gm*	*γ per gm*
Yeast extract	11.2	14.5	9.1	10.0	1.4	1.3
Liver "	8.1	15.9	13.0	22.5	0.47	1.2
Vitab	20.4	130	3.0	3.0	0.43	2.2
Grass juice	10.7	16.5	9.0	11.5	2.2	4.5

Examination of Table I reveals that with liver and yeast extracts and grass juice only a small fraction (one-fourth to one-tenth) of the total active substances present, as indicated by yeast assay, behaves as does pyridoxal; *i.e.*, is active for *Lactobacillus casei*. This portion of the total will subsequently be called the *pyridoxal fraction*. When the activity contributed by the pyridoxal fraction to the growth of *Streptococcus faecalis* is subtracted from the total assay value obtained with this organism, a figure is obtained for the amount of material present which behaves as does pyridoxamine for this organism (the *pyridoxamine fraction*). If the sum of the activities contributed by the pyridoxal and pyridoxamine fractions is subtracted from the figure obtained by yeast assay, the result gives the amount of material present which behaves as pyridoxine toward the three test organisms.[2] By inspection, it is apparent that with yeast and liver extracts and with grass juice most of the active material is in the pyridoxamine fraction, with relatively much lower amounts in the pyridoxal and pyridoxine fractions. With vitab, the pyridoxine fraction far surpasses the other two. It should be pointed out that the first three of the above materials are extracts of metabolically active tissues, whereas the latter is derived primarily from dormant tissue.

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[1] Such values are readily obtained for comparison by dividing the assay figures for the substance in question by the figure given for the activity of pyridoxine Thus, yeast extract, assayed with *Streptococcus faecalis*, contains the equivalent of 20,000 γ of pyridoxine per gm or 20,000 "γ equivalents" per gm. as originally designated (1).

Utilization of Pyridoxamine and Pyridoxal Fractions by Yeast—Division of the total vitamin B_6 activity of natural materials for yeast into three fractions, as described above, rests upon the assumption that the pyridoxamine and pyridoxal fractions are utilized for growth of yeast, as are pyridoxamine and pyridoxal themselves (6). These fractions together constitute what was previously called "pseudopyridoxine." Various investigators (7, 9–12), however, using yeasts, molds, or rats as test organisms, have concluded that "pseudopyridoxine" is inactive for these organisms.

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[2] It is tempting to apply quantitative treatment to the results. This works fairly successfully with mixtures of the three pure compounds Thus, with values for the activity of compounds as reported in Table I, the following equations can be used for *Lactobacillus casei* (pyridoxal standard) $T = x + 0.0023y + 0.0008z$, for *Streptococcus faecalis* (pyridoxamine standard) $T = 0.36x + y + 0.0005z$, for *Saccharomyces carlsbergensis* (pyridoxine standard) $T = 1.24x + 1.2y + z$, where T is the total assay value obtained with each organism as stated, and x, y, and z are the concentrations of pyridoxal, pyridoxamine, and pyridoxine, respectively Because of the low activity of pyridoxamine and pyridoxine for *L casei*, and the low activity of pyridoxine for *S. faecalis*, these equations reduce to $T = x$ (*L casei*), $T = 0.36x + y$ (*S. faecalis*), $T = 1.24x + 1.2y + z$ (*S carlsbergensis*) The coefficients are somewhat variable, and must be determined with each test. Unfortunately, the assays involved are not very accurate, especially when applied to natural materials. As an illustration of the use of the method, it may be applied to the results on acid-hydrolyzed yeast extract (Table I). Here, the pyridoxal fraction (x) is 1.3 γ per gm., the pyridoxamine fraction (y) is 9.5 γ per gm , and the pyridoxine fraction is 1.5 γ per gm. The total error in the three determinations is borne by the pyridoxine fraction. The figure for this fraction is meaningless in this instance, since it amounts to little more than 10 per cent of the total assay figure for yeast, whereas none of the assays used are accurate to more than ±10 to 15 per cent. For purposes of this paper, it is unnecessary to solve such equations in detail. The relative amounts of the pyridoxamine and pyridoxal fractions present can be seen by mere inspection of the comparative assay figures obtained with *S. faecalis* and *L. casei*.

With one exception (12), this conclusion was based on the observed facts that substances known to contain "pseudopyridoxine" failed to exhibit abnormally high assay values with these organisms when assayed against a pyridoxine standard, or upon failure of autoclaving pyridoxine with amino acids to increase its growth-promoting activity for the organism in question. As Johnson (12) points out, however, if pyridoxine and "pseudopyridoxine" have the *same* activities for an organism, abnormally high assay values would not be expected. In Paper III (6) it was shown that pyridoxine, pyridoxamine, and pyridoxal actually have approximately equal activities for each of the organisms used by these workers. In the present paper, fairly conclusive evidence will be given that "pseudopyridoxine" consists principally, if not entirely, of a mixture of pyridoxal and pyridoxamine or higher combinations of these compounds, and is, therefore, available for the growth of all organisms so far examined. Johnson (12), however, has presented evidence that "pseudopyridoxine" is not available for the growth of yeast (*Saccharomyces carlsbergensis*). He used *Lactobacillus casei* as the assay organism; his conclusions apply, therefore, only to the pyridoxal fraction of the total "pseudopyridoxine" activity. Since his conclusions are at variance with those reported herein, an experiment similar to that he reports was carried out.

TABLE II

Utilization of Pyridoxamine and Pyridoxal Fractions of Yeast Extract by Saccharomyces carlsbergensis

Flask No	Additions	Inoculated	Total vitamin B_6 in eluate as determined with		
			Saccharomyces carlsbergensis (pyridoxine standard)	*Streptococcus faecalis* (pyridoxamine standard)	*Lactobacillus casei* (pyridoxal standard)
			γ	γ	γ
1	0.4 γ pyridoxine hydrochloride	No	0.28	*	*
2	0.4 " " "	Yes	0.0062		
3	25 mg. yeast extract	No	0.21	0.24	0.011
4	25 " " "	Yes	0.015	0.018	0.0035

* Since pyridoxine is essentially inactive for these organisms, no assay figure was obtained

Four 1000 cc. flasks, each containing 200 cc. of the pyridoxine assay medium of Atkin *et al.* (7), were prepared. 0.4 γ of pyridoxine hydrochloride was added to Flasks 1 and 2. This is sufficient pyridoxine to permit about one-half maximum growth of *Saccharomyces carlsbergensis* (7). An amount of yeast extract (25 mg.) calculated to contribute an equivalent amount of total vitamin B_6 was added to Flasks 3 and 4. After sterilization, Flasks 2 and 4 were inoculated with *S. carlsbergensis;* Flasks 1 and 3 were carried as controls. All flasks were then shaken at 32° for 20 hours. At the end of this time, Flasks 2 and 4 showed heavy growth of yeast; Flasks 1 and 3 were still sterile. The contents of all flasks were then filtered through thin layers of Filter-Cel, and 0.5 gm. of Lloyd's reagent added to each filtrate. After the flasks were shaken for 20 minutes, the Lloyds reagent from each flask was separately filtered, washed with water, and eluted with 10 cc. of 0.5 N NaOH (two 5 cc. portions). Each eluate was neutralized and assayed with the three test organisms. The results are given in Table II. They indicate that about 98 per cent of the pyridoxine originally present in Flask 2 was absorbed by the growing yeast. 92.8 per cent of the vitamin B_6 originally present in the yeast extract added to Flask 4 was absorbed, as indicated by yeast assay. Similarly, 92.7 per cent was absorbed as indicated by assay with *Streptococcus faecalis*, which responds only to "pseudopyridoxine" (pyridoxamine plus pyridoxal fractions). The quantitative agreement between results with *S. carlsbergensis* and *Streptococcus faecalis* is not merely coincidental. Data of Table I, and that to be reported later, indicate that pyridoxamine is the chief compound with vitamin B_6 activity which occurs in yeast extract. This must therefore be the principal compound absorbed by yeast. Assay with *Lactobacillus casei* showed absorption of 68 per cent of the pyridoxal fraction originally present. Thus both the pyridoxamine and pyridoxal fractions of "pseudopyridoxine" are available for yeast growth, and hence division of the total vitamin B_6 activity of natural products for yeast into pyridoxine, pyridoxamine, and pyridoxal fractions appears justified. The lowered percentage absorption of the pyridoxal as compared with the pyridoxamine fraction does not mean that the pyridoxal fraction is less readily utilized, but is merely a reflection of the comparatively low concentration in which it is originally present, for if assimilation by an organism depends upon adsorption and diffusion processes, it is clear that, other things being equal, substances present in comparatively high concentration will be preferentially utilized.[3]

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[3] If a similar distribution of the pyridoxamine and pyridoxal fractions occurs in sweat, a possible explanation of the results of Johnson is afforded. It is possible, of course, that compounds occur in sweat that are active for *Lactobacillus casei* and not for yeast, but this is not considered likely. The properties of the active substance present in sweat, so far as they are reported (12), fit those of pyridoxal. Thus the activity of sweat decreased from 10 γ equivalents to 0 13 γ equivalent of pyridoxine per cc. when the test was autoclaved for 20 instead of for 10 minutes This was ascribed to increase in the activity of the pyridoxine standard during autoclaving, but in our experience increases of this magnitude do not occur when *L. casei* is the test organism (*cf.* Table V and also (6)). The decrease is more likely due

Nitrous Acid Treatment—Pyridoxine is stable to nitrous acid (13), while one would expect pyridoxal also to be stable. Pyridoxamine, however, should be completely destroyed by nitrous acid, and one of the principal products of the decomposition should be pyridoxine. If pyridoxamine is responsible for activity of the pyridoxamine fraction of natural materials, the latter should be inactivated by nitrous acid treatment.

The effect of nitrous acid was checked as follows: To separate 25 cc. flasks containing 2 mg. portions of pyridoxine, pyridoxamine, or pyridoxal dissolved in 2 cc. of water, there were added 0.5 cc. of glacial acetic acid and 1 cc. of a solution containing 20 mg. of sodium nitrite. The flasks were shaken for 30 minutes, then 50 mg. of urea were added per flask, and shaking was continued for 30 minutes. Solutions of yeast extract, liver extract, vitab, and dehydrated grass juice were hydrolyzed as indicated previously, neutralized, and similarly treated. To 2 cc. aliquots of the hydrolysates placed in separate flasks and containing 100 mg. of the product in question, there were added 2.5 cc. of a solution containing 50 mg. of sodium nitrite and 0.5 cc. of glacial acetic acid. After 30 minutes shaking, 100 mg. of urea were added, and the shaking continued for 30 minutes. The contents of all flasks were then neutralized and diluted for assay. The results are shown in Table III.

TABLE III

Effect of Treatment with Nitrous Acid on Vitamin B_6 Activity of Various Substances

Substance	Assay					
	Saccharomyces carlsbergensis (pyridoxine standard)		*Streptococcus faecalis* (pyridoxamine standard)		*Lactobacillus casei* (pyridoxal standard)	
	Untreated	HNO$_2$-treated	Untreated	HNO$_2$-treated	Untreated	HNO$_2$-treated
	γ *per* γ	γ *per* γ	γ *per* γ	γ *per* γ	γ *per* γ	γ *per* γ
Pyridoxine	1.0	1.1	0.0025	0.0030	0.00076	0.00080
Pyridoxamine	1.1	0.47	1.0	0.0011	0.0070	0.0030
Pyridoxal	1.1	1.1	0.36	0.34	1.0	1.0
	γ *per gm*	γ *per gm*	γ *per gm*	γ *per gm*	γ *per gm*	γ *per gm*
Yeast extract	13.0	5.5	11.7	0.39	1.0	1.3
Liver "	9.1	6.3	16.2	1.2	0.8	1.4
Vitab	92.0	86.0	3.0	1.7	4.7	4.2
Grass juice	11.7	6.6	6.8	1.5	5.6	5.6

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to inactivation of pyridoxal by heating with the hydrolyzed casein present in the medium (*cf.* Table V). That such inactivation of the substance active for *L. casei* occurs was not recognized at the time the experiments with sweat were carried out. Chance variations in assay due to small variations in autoclaving technique could well explain failure to observe utilization of the substance by yeast.

Pyridoxine and pyridoxal were unaffected by nitrous acid treatment. Pyridoxamine was completely decomposed, as judged from its growth effect on *Streptococcus faecalis*. Somewhat less than 50 per cent was converted to pyridoxine, as determined by its residual growth activity for yeast. The behavior of natural extracts is exactly what would be predicted if pyridoxal and pyridoxamine were alone responsible for activity of the pyridoxal and pyridoxamine fractions. Thus there is no decrease in the pyridoxal activity following nitrous acid treatment. The pyridoxamine fraction is completely inactivated. The residual activity remaining for *Streptococcus faecalis* after nitrous acid treatment is about that to be expected from the undestroyed pyridoxal fraction. The lowered result obtained on yeast assay following nitrous acid treatment is about what would be expected if pyridoxamine accounted for a large portion of the total activity (for yeast, liver, and grass extracts), and this pyridoxamine, like the pure control compound, was incompletely converted to pyridoxine by the treatment given. Finally, the active substance in vitab for yeast, previously seen to fall principally in the pyridoxine fraction, is unaffected by nitrous acid. Adequate toxicity controls were run with each assay to insure that any lowered assay results obtained were not due to possible toxic materials remaining after nitrous acid treatment.

Thus the behavior of natural materials toward nitrous acid is consistent with the hypothesis that pyridoxamine and pyridoxal occur naturally, and account for the greater fraction of the vitamin B_6 activity of many products.

Treatment with Sodium Cyanide and Ammonium Chloride—It has been shown (3) that when crude oxidation mixtures derived from pyridoxine were incubated with cyanide and ammonium chloride, the pyridoxal contained therein was inactivated, presumably because it was changed to the cyanhydrin or an amino nitrile. Activity of amination products of pyridoxine (pyridoxamine) was unaffected by this treatment. If the activity of the pyridoxal fraction of natural materials is actually due to pyridoxal, activity of such materials should be greatly decreased for *Lactobacillus casei* by incubation with cyanide and ammonium chloride. Since the pyridoxal fraction is small in comparison with the pyridoxamine fraction, only a small decrease in activity for *Streptococcus faecalis* would be expected, if activity of the latter fraction is actually due to pyridoxamine. Similarly, relatively small changes in activity for yeast would be expected.

1 mg. portions of pyridoxine, pyridoxamine, or pyridoxal were dissolved with 20 mg. of sodium cyanide and 20 mg. of ammonium chloride in 5 cc. of water. 100 mg. portions of the acid-hydrolyzed, natural materials were neutralized, 20 mg. of sodium cyanide and 20 mg. of ammonium chloride added, and the volume adjusted to 5 cc. All tubes were allowed to stand

overnight at room temperature, then diluted to 10 cc., pasteurized at 100° in the steamer for 5 minutes, and diluted for assay.

The per cent inactivation for each organism is shown in Table IV. Pyridoxine and pyridoxamine were unaffected by the treatment; pyridoxal was rendered almost completely inactive for all organisms. The changes in the activity of natural materials were exactly those outlined above: the pyridoxal fraction (active for *Lactobacillus casei*) was over 90 per cent destroyed, while only minor changes occurred in the activity as determined with *Streptococcus faecalis* and yeast.[4] Again the behavior of natural materials is in accord with the supposition that these compounds occur naturally.

TABLE IV

Effect of Treatment with Sodium Cyanide and Ammonium Chloride or with Acetone and Alkali on Vitamin B_6 Activity of Various Substances

Substance	Per cent of activity destroyed by incubation with					
	Cyanide and ammonium chloride			Acetone and sodium hydroxide		
	Saccharomyces carlsbergensis	*Streptococcus faecalis*	*Lactobacillus casei*	*Saccharomyces carlsbergensis*	*Streptococcus faecalis*	*Lactobacillus casei*
Pyridoxine	0.0	0.0	0.0	0.0	0.0	0.0
Pyridoxamine	0.0	0.0	0.0	0.0	0.0	0.0
Pyridoxal	98	99	98	98	98	97
Yeast extract	11	6.0	88	15	9.0	54
Liver "	25	0.00	93	6.7	11	87
Vitab	3.6	4.3	90	0.0	39	80
Grass juice	9.4	18	92	15	10	90

Effect of Incubation with Acetone and Alkali—Pyridoxal is inactivated by standing with acetone in alkaline solution; pyridoxamine is not (3). If these substances occur naturally, activity for *Lactobacillus casei* should be largely destroyed by this treatment, while that for yeast and *Streptococcus faecalis* should be only slightly and variably decreased, depending upon the fraction of the total activity contributed by pyridoxal. 2 mg. portions of the crystalline vitamins or 100 mg. portions of the natural products were mixed with 1.0 cc. of acetone in 5 cc. of 0.4 N sodium hydroxide.

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[4] Because the pyridoxal fraction is usually so small compared to the pyridoxamine fraction, and since the assays are accurate to only ±10 to 15 per cent, little significance can be attached to the decreases in activity tabulated for natural products under *Streptococcus faecalis* and *Saccharomyces carlsbergensis*. There is a consistent decrease, however, of a magnitude approximately in line with the portion of the total activity comprised by the pyridoxal fraction, as indicated in Table I

After standing at room temperature for 4 hours, samples were neutralized and diluted for assay.

The results given in Table IV are consistent with the view that pyridoxamine and pyridoxal occur naturally. For *Lactobacillus casei*, activity of natural products is from 50 to 90 per cent destroyed. For *Streptococcus faecalis*, only small losses occur,[4] except in the case of vitab. Reference to Table I, however, shows that a considerable portion of the total activity of vitab for *Streptococcus faecalis* is comprised by the pyridoxal fraction, so that this decrease should be expected. For yeast, as would be expected, only minor losses in activity occur.[4]

The rate and extent of inactivation of the pyridoxal fraction of natural materials by this treatment are less than those exhibited by pure pyridoxal. This may indicate that a portion of the pyridoxal which occurs naturally is in higher combinations which partially protect it from reaction, or that other constituents of the extracts influence the rate and extent of the reaction.

Effect of Heating with Casein—It was previously reported that the activity of pyridoxal for *Lactobacillus casei* was very largely destroyed by heating with a casein hydrolysate (6). The same treatment increased the activity of pyridoxal for *Streptococcus faecalis*, and left it unchanged for yeast. The activity of pyridoxamine for all three organisms was unchanged by the same treatment (2, 6). Autoclaving natural materials with hydrolyzed casein should, therefore, result in greatly decreased activity for *Lactobacillus casei*, and slightly increased activity for *Streptococcus faecalis*, if these compounds occur naturally.

1 mg. portions of pyridoxine, pyridoxamine, or pyridoxal, and 100 mg. portions of the acid-hydrolyzed natural materials were added to separate 1800 mg. portions of casein hydrolysate (vitamin-free). All solutions were at pH 6.9 and were adjusted to a volume of 10 cc. One set of these solutions was autoclaved at 15 pounds pressure for 1 hour. An identical set was prepared and held at room temperature as controls

The changes in activity of the various products which occurred as a result of heating with casein are given in Table V. Results with the pure compounds are the same as those previously reported. The activity of pyridoxal is 96 per cent destroyed for *Lactobacillus casei* and considerably increased for *Streptococcus faecalis* by the treatment. Similarly, 60 to 80 per cent of the activity of natural materials for *L. casei* (pyridoxal fraction) is destroyed by the treatment, while the activity for *Streptococcus faecalis* is correspondingly increased. In separate, similar experiments, with yeast as the assay organism, only minor changes in activity occurred, for the most part no greater than could be accounted for by the experimental error of the method. Separate experiments showed that the rate at which pyridoxal was inactivated for *L. casei* by this treatment was greater than the rate at which the pyridoxal fraction of natural materials was inactivated. Again, this may mean simply that other constituents of natural materials influence the rate and extent of the reaction, or it may indicate that pyridoxal occurs naturally in some combined form less susceptible to this type of change.

Effect of Heating with α-Ketoglutaric Acid[5]—It was originally suggested (4) that pyridoxamine and pyridoxal might be interconvertible by transamination reactions. The inactivation of pyridoxal for *Lactobacillus casei*, and the accompanying increase in activity for *Streptococcus faecalis*, noted above when pyridoxal was heated with hydrolyzed casein (*cf.* also (6)) are most simply explained by assuming that pyridoxal is transformed into pyridoxamine by transamination reactions. Unpublished experiments show that this effect of casein is largely due to glutamic acid, which is known to be involved in biological transaminations (14, 15). This suggests attempting the reverse reaction; *i.e.*, transformation of pyridoxamine into pyridoxal by heating with α-ketoglutaric acid. This subject will be separately treated at a later date. Results of a preliminary experiment, however, are shown in Table VI. 10 mg. portions of the hydrolyzed natural materials were mixed with 5 mg. portions of α-ketoglutaric acid. 100 γ portions of the crystalline vitamins were similarly mixed with 500 γ of α-ketoglutaric acid. All solutions were adjusted to pH 7.0 and 2 cc. and heated in the autoclave at 15 pounds pressure for 30 minutes.

TABLE V

Effect of Heating with Hydrolyzed Casein on Vitamin B_6 Activity of Various Substances

Substance	Assay with			
	Streptococcus faecalis (pyridoxamine standard)		*Lactobacillus casei* (pyridoxal standard)	
	Unheated	Heated	Unheated	Heated
	γ per γ	*γ per γ*	*γ per γ*	*γ per γ*
Pyridoxine	0.00042	0.025	0.0011	0.0053
Pyridoxamine	1.00	1.00	0.019	0.020
Pyridoxal	0.23	0.87	1.00	0.036
	γ per gm	*γ per gm*	*γ per gm*	*γ per gm.*
Yeast extract	8.5	12.0	1.1	0.44
Liver "	12.8	15.3	1.1	0.37
Vitab	2.4	3.4	1.8	0.45
Grass juice	6.5	9.0	4.5	0.87

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[5] The α-ketoglutaric acid used was generously supplied by Dr. F. Schlenk.

As would be expected if pyridoxamine were actually transformed in part to pyridoxal, its activity was greatly increased for *Lactobacillus casei*, considerably decreased for *Streptococcus faecalis*, and essentially unchanged for yeast. The activities of pyridoxal and pyridoxine were not affected by this treatment. If pyridoxamine occurs naturally, increased activity for *Lactobacillus casei* should follow treatment of natural materials with α-ketoglutaric acid, while activity for *Streptococcus faecalis* should be somewhat decreased. No change (beyond the ±10 per cent accuracy of the assay) should occur in activity for yeast In general, exactly these trends are observed. It is obvious that substances other than pyridoxamine, present in the natural materials in large amounts, may prevent the changes observed from being as large as might be expected from the results with the pure substance.

TABLE VI

Effect of Heating with α-Ketoglutaric Acid on Vitamin B_6 Activity of Various Substances

Substance	Assay with					
	Saccharomyces carlsbergensis (pyridoxine standard)		*Streptococcus faecalis* (pyridoxamine standard)		*Lactobacillus casei* (pyridoxal standard)	
	Untreated	Treated	Untreated	Treated	Untreated	Treated
	γ per γ	*γ per γ*	*γ per γ*	*γ per γ*	*γ per γ*	*γ per γ*
Pyridoxine	1.00	1.14	0.00047	0.00036	0.00075	0.00088
Pyridoxamine	0.87	0.88	1.00	0.54	0.0032	0.48
Pyridoxal	0.95	0.92	0.40	0.39	1.00	1.00
	γ per gm	*γ per gm*	*γ per gm.*	*γ per gm.*	*γ per gm*	*γ per gm*
Yeast extract	13.7	13.9	14.5	9.5	2.2	7.7
Liver "	17.6	19.4	23.0	16.0	3.2	4.9
Vitab	125	105	3.7	2.1	3.9	5.4
Grass juice	15.0	16.5	11.5	8.0	4.5	8.3

DISCUSSION

In all reactions attempted, the pyridoxamine fraction of natural materials behaves as though pyridoxamine itself was the active substance present. Similarly, the pyridoxal fraction of natural materials behaves as though pyridoxal was chiefly responsible for its activity. These pure compounds are available for growth of yeast, and of other organisms which utilize pyridoxine, as well as for many organisms unable to utilize pyridoxine (6). Similarly, the pyridoxamine and pyridoxal fractions of natural materials, previously called "pseudopyridoxine," are available for the growth of yeast, and are utilized by those organisms which cannot utilize pyridoxine. The simplest explanation for data so far available is to assume that these two compounds occur naturally and constitute a considerable portion of the total vitamin B_6 activity of natural materials. Just as thiamine, for example, occurs both free and combined as cocarboxylase in nature, so it is possible that higher combinations of pyridoxal and pyridoxamine occur naturally, and contribute to the activity of the pyridoxal or pyridoxamine fractions for various organisms. The existence of inactive conjugates of these substances, similar to that reported by Scudi *et al.* (16) for pyridoxine, is probable, since assay values of some products are increased by acid hydrolysis.

By means of the assays used above, it is possible to determine roughly the portion of the total vitamin B_6 activity which is due to pyridoxal and to pyridoxamine. With some products, such as liver and yeast extracts, these account for the greatest share of the vitamin B_6 activity. With other products, such as rice bran concentrate, only very small amounts of pyridoxamine and pyridoxal occur, as compared to the amount of pyridoxine present. When the pyridoxamine and pyridoxal fractions constitute a major portion of the total activity, the differential assay used is not sufficiently accurate to determine the amount of pyridoxine actually present.

Recently, two studies have appeared which support the contention that pyridoxal occurs naturally. Huff and Perlzweig (17) have shown that 2-methyl-3-hydroxy-4-carboxy-5-hydroxymethylpyridine (4-pyridoxic acid) is excreted to a variable extent by various animals after ingestion of pyridoxine The growth-promoting activity of this product has been previously reported (6). This demonstrates that animals oxidize pyridoxine at the 4-hydroxymethyl group. Pyridoxal is a logical intermediate which would be expected to arise during the course of such an oxidation. Gunsalus and Bellamy (18) have shown that "pseudopyridoxine" promotes decarboxylation of tyrosine by resting cells of *Streptococcus faecalis*. Pyridoxal was tested and found active Natural products contain a similarly active substance (19).

Very recently Hochberg *et al.* (20), using a chemical method specific for pyridoxine, have shown that some products, notably yeast extract, do not contain enough pyridoxine to account for their vitamin B_6 activity, as determined biologically. Their data with yeast extract thus agree with those reported herein. Neither pyridoxamine nor pyridoxal is determined by their procedure.[6] Their data also support ours in the finding that rice bran extract contains chiefly pyridoxine.

SUMMARY

A differential assay method is described with *Lactobacillus casei*, *Streptococcus faecalis*, and *Saccharomyces carlsbergensis* by means of which substances present in natural materials which promote growth of these organisms on

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[6] Melnick, D , personal communication

appropriate vitamin B_6-free media can be classified into three fractions which resemble pyridoxal, pyridoxamine, or pyridoxine in growth-promoting properties. Behavior of these fractions of natural materials to various chemical treatments was then determined, and compared with the behavior of the pure compounds.

Pyridoxal, like pyridoxine, was undamaged by nitrous acid treatment. Similarly, the pyridoxal fraction of natural materials was unaffected. Pyridoxamine and the pyridoxamine fraction of natural materials are completely inactivated by nitrous acid. The activity of pyridoxamine for yeast is only partially destroyed by this treatment, since one product of the reaction is pyridoxine. Similarly, the activity of natural materials for yeast is only partially destroyed.

Pyridoxal and the pyridoxal fraction of natural materials are almost completely inactivated by incubation with sodium cyanide and ammonium chloride; pyridoxamine and the pyridoxamine fraction of natural materials are unaffected by this treatment.

Pyridoxal and the pyridoxal fraction of natural materials are largely inactivated by incubation with alkali and acetone. Pyridoxamine and the pyridoxamine fraction of natural materials are unaffected by the treatment. The rate and extent of inactivation of the pyridoxal fraction are somewhat less than that which occurs with pure pyridoxal.

Pyridoxal and the pyridoxal fraction of natural materials are largely inactivated by autoclaving with hydrolyzed casein. This treatment increases the activity of pyridoxal for *Streptococcus faecalis*, as though pyridoxamine was being formed. Similarly, the activity of the pyridoxamine fraction of natural materials is increased by this treatment.

Autoclaving pyridoxamine with α-ketoglutaric acid greatly increases its activity for *Lactobacillus casei*, but decreases it for *Streptococcus lactis*. Both changes are consistent with the interpretation that pyridoxamine is being converted to pyridoxal. On similar treatment of natural products, the activity of the pyridoxal fraction is increased and that of the pyridoxamine fraction is decreased.

For yeast, pyridoxal, pyridoxamine, and pyridoxine have equal activities. Changes in activity of natural materials resulting from the above treatments are exactly those which would be expected from the behavior of the three pure compounds. Just as yeast can utilize pyridoxamine and pyridoxal for growth, it is shown that the pyridoxamine and pyridoxal fractions of natural materials ("pseudopyridoxine") are also utilized by yeast.

It is concluded that pyridoxal and pyridoxamine occur naturally, that they constitute a considerable portion of the total vitamin B_6 present in many natural materials, and that the proportions in which the three compounds occur differ markedly with different materials. The presence of these compounds satisfactorily explains "pseudopyridoxine" activity (1). The data do not exclude the possibility that these substances may occur to some extent as constituent parts of more complex structures, nor are they sufficiently quantitative to exclude the possibility that other unknown substances may contribute a minor portion of the total vitamin B_6 activity of natural materials.

BIBLIOGRAPHY

1. Snell, E. E , Guirard, B. M., and Williams, R. J., *J. Biol. Chem.*, **143,** 519 (1942).
2. Snell, E. E., *Proc. Soc. Exp. Biol. and Med.*, **51,** 356 (1942).
3. Snell, E. E., *J. Am. Chem. Soc.*, in press.
4. Snell, E. E., *J. Biol. Chem.*, **154,** 313 (1944).
5 Harris, S. A , Heyl, D., and Folkers, K , *J. Biol. Chem.*, **154,** 315 (1944); *J. Am. Chem. Soc.*, in press.
6 Snell, E. E , and Rannefeld, A. N , *J. Biol. Chem.*, **157,** 475 (1945).
7 Atkin, L , Schultz, A S., Williams, W. L., and Frey, C. N., *Ind. and Eng. Chem., Anal Ed.*, **15,** 141 (1943).
8. Siegel, L., Melnick, D., and Oser, B. L., *J. Biol. Chem* , **149,** 361 (1943).
9. Robbins, W J., and Ma, R., *Proc. Nat. Acad. Sc.*, **29,** 172 (1942).
10 Carpenter, L E , Elvehjem, C A , and Strong, F. M , *Proc. Soc. Exp. Biol. and Med* , **54,** 123 (1943).
11 Stokes, J L., Larsen, A., Woodward, C. R., Jr., and Foster, J. W., *J. Biol. Chem.*, **150,** 17 (1943).
12. Johnson, B C , *Proc. Soc. Exp. Biol. and Med.*, **55,** 199 (1944).
13. Stiller, E. T., Keresztesy, J. C., and Stevens, J. R., *J. Am. Chem. Soc.*, **61,** 1237 (1939).
14. Braunstein, A E , *Enzymologia*, **7,** 25 (1939).
15. Cohen, P P., *Biochem J* , **33,** 1478 (1939).
16. Scudi, J. V., Buhs, R. P., and Hood, D. B., *J. Biol. Chem.*, **142,** 323 (1942).
17. Huff, J. W , and Perlzweig, W A , *J. Biol. Chem* , **155,** 345 (1944).
18. Gunsalus, I C , and Bellamy, W. D., *J. Bact* , **47,** 413 (1944).
19. Gunsalus, I C., and Bellamy, W. D., *J. Biol. Chem.*, **155,** 357 (1944).
20. Hochberg, M., Melnick, D , and Oser, B. L., *J. Biol. Chem.*, **155,** 119 (1944).

COLORIMETRIC DETERMINATION OF UREA

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The use of α-isonitrosopropiophenone, $C_6H_5 \cdot CO \cdot CNOH \cdot CH_3$, as a reagent for the colorimetric determination of urea has been mentioned in a previous communication (1). The present report outlines a simple method for the colorimetric determination of urea in blood filtrates and urine by means of this reagent and compares the values obtained by this method with those given by the urease ((2), (3) p. 372) methods.

Apparatus

Spectrophotometer or *colorimeter.*

Reaction tubes. These are test-tubes of Pyrex glass, 20 by 150 mm. Each tube is fitted with a 1-hole rubber stopper through which is passed a short heavy walled glass capillary tube of 0.5 to 1.0 mm. bore. Such tubes have been illustrated by Van Slyke and Hawkins (4). These tubes are used to hold the blood filtrate and other urea solutions during the chromogenic reaction with α-isonitrosopropiophenone in the hot water bath.

Reagents

Sulfuric-phosphoric acid mixture (1). 1 volume of concentrated sulfuric acid, 3 volumes of syrupy phosphoric acid, and 1 volume of water.

α-Isonitrosopropiophenone.[1] 4 gm. in 100 cc. of alcohol.

Stock standard solution of urea. 10.7 mg. of urea in 100 cc. of water. Stored in the ice box; prepared fresh monthly.

Working standard solution. 0.0107 mg. of urea per cc., containing 0.005 mg. of urea N per cc. To prepare the working standard, 1 cc. of stock standard is diluted to 10 cc. with water.

Acetate buffer. 10 gm. of sodium acetate $NaC_2H_3O_2 \cdot 3H_2O$ and 10 cc. of glacial acetic acid made up to 1 liter with water.

Procedure

Preparation of Blood Filtrates—Filtrates prepared according to the method of Somogyi (5), Fujita and Iwatake (6), or Miller and Van Slyke (7) are suitable.[2] Dialysates prepared according to the technique of Hamilton and Archibald (9) are equally satisfactory. 2 cc. aliquots of a 1:10 filtrate or dialysate are used when the blood urea level is within normal limits. For uremic blood greater dilution than 1:10 is used. Each 2 cc. portion is measured into a reaction tube and mixed with exactly 5 cc. of water.

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[1] Obtained from the Eastman Kodak Company, or from Anachemia, Ltd., Montreal, Canada, and 70 East 45th Street, New York 17.

Preparation of Urine—Urine of adults is diluted according to the volume excreted per minute, as shown in Table I. For children the observed urine flow is corrected for the size of the patient, according to the directions of McIntosh, Møller, and Van Slyke (10), and the corrected volume per minute is applied to Table I. The urines can be diluted in large graduated cylinders with as great an accuracy as the rate of urine flow is ordinarily measured.

Urines containing more than 0.5 per cent protein should be cleared of protein before dilution. Urine containing 0.5 per cent protein after dilution to 1:500 contains 0.01 mg. per cc. This amount of protein causes an error of +1 per cent. To free urine of protein, add to 5 cc. an equal volume of acetate buffer. The mixture then has a pH between 4 and 5. The urine is brought to 100° and the precipitated protein is removed by centrifugation or filtration. The protein-free solution is then diluted, as indicated in the last column of Table I, to give the urine dilution indicated by the middle column.

Of the diluted urine 2 cc. are measured into a reaction tube and mixed with 5 cc. of water.

Thymol should not be used as a urine preservative, because if more than 0.001 gm. is present in the final solution analyzed it causes turbidity.

Preparation of Reagent Blank and Standards—Reagent blank and standards are prepared by adding to reaction tubes 0, 2, 4, and 6 cc. portions of working standard and 7, 5, 3, and 1 cc., respectively, of water.

Development of Color—To the 7 cc. of fluid in each tube of blood filtrate, dilute urine, blank, or standard, add 5 cc. of the sulfuric-phosphoric acid mixture and 0.40 cc. of the alcoholic solution of α-isonitrosopropiophenone. The contents of each tube are mixed thoroughly. The tubes are then closed with their perforated rubber stoppers and are placed in a boiling water bath from which light is excluded. The water in the bath should come just above the level of liquid in the tubes After exactly 1 hour of heating the tubes are set in a covered kettle containing water at room temperature. A large kitchen pot, the lid and inside of which have been painted black, serves well. After 15 minutes, readings are taken in a colorimeter or in a spectrophotometer set at wave-length 540 mμ with the optical density of the reagent blank adjusted to zero. When a visual colorimeter is used, it is important to select a standard which has a color depth almost equal to that of the sample.

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[2] The amount of urea in the Folin-Wu (8) filtrate has been shown by urease measurement to be no less than in the other filtrates. Nevertheless when Folin-Wu (8) filtrates of dog whole blood are compared with standards to which no sodium tungstate or sodium sulfate is added, the values obtained for urea by the colorimetric method average 10 per cent lower than values obtained with the other filtrates or with the dialysate. For this reason Folin-Wu filtrates are not recommended for use in the colorimetric determination of urea.

Calculations

When the photometer is used, a curve is constructed by plotting the mg. of urea in the standards against the corresponding optical densities. From the optical densities of the samples the mg. of urea (P) in each are read off from the curve.

TABLE I

Relation of Urine Flow to Dilution Required

Corrected urine flow	D = dilutions for urines containing less than 0 5 per cent protein	Dilutions for urine filtrate after coagulation of protein
cc per min		
<0.2	1:1000	1:500
0.2- 0.5	1·750	1:375
0.5- 1.0	1 500	1:250
1.0- 2.0	1:300	1:150
2.0- 4.0	1:200	1:100
4.0- 9.0	1:120	1:60
9.0-15.0	1:50	1:25

$$\text{Mg urea N per 100 cc blood or urine} = 50DP$$

where D = the number of volumes to which 1 volume of blood or untreated urine was diluted.

When a visual colorimeter is used

$$\text{Mg. urea N in sample} = C \times \frac{S}{U}$$

where S = the reading of the standard, U = the reading of the unknown; and C = the number of mg. of urea N in the standard.

$$\text{Mg. urea N per 100 cc. blood or urine} = 50DC \times \frac{S}{U}$$

where D = the number of volumes to which 1 volume of blood or untreated urine was diluted.

In the case of normal blood $D = 10$ and $C = 0.03$. Hence

$$\textit{Mg. urea N per 100 cc. blood} = 15\,\frac{S}{U}$$

Mg. of urea N are converted to mg. of urea by the use of the factor 2.143.

Discussion of Method

The color produced by reaction of urea with α-isonitrosopropiophenone in acid is red. The structure of the product is unknown. As is the case in the diacetyl-carbamido reaction (1), the color obtained is photolabile. Hence it is necessary to keep the tubes in the dark during and after heating until they are read. The color obtained under the prescribed conditions is stable in the dark for more than 24 hours. After 24 hours, less than 0.5 per cent of the color is faded.

The advantages of α-isonitrosopropiophenone over diacetylmonoxime for determination of urea are (1) the reagent is less volatile; (2) the product has a red rather than a yellow color and is more readily compared by the eye; (3) the reagent is relatively less sensitive to citrulline and other urea derivatives than is diacetylmonoxime; therefore it is more specific for urea.

For some purposes it may be desirable to reduce the time of heating. When large concentrations of allantoin are present (as in the case of urine other than human), the specificity of the method can be increased by reducing the heating period to 20 minutes. Under these conditions, although only 27 per cent as much color is obtained with urea, only 4 per cent as much color is obtained with allantoin (see Fig. 3) as when the heating is for 60 minutes. The specificity of the reaction in the presence of allantoin can be further increased by reducing the concentration of sulfuric acid in the acid mixture (1). Lower dilutions of urine or blood will be required when these alternative conditions are employed.

Results

The optical density curves of the colored products obtained with urea and blood filtrate under the conditions of analysis outlined above are given in Fig. 1. It will be noted that the curves yielded by blood filtrates are practically identical with curves yielded by pure urea solutions of similar urea content. This close approximation to identity is evidence in favor of the probability that no significant part of the optical density in the utilized part of the curve obtained with blood filtrate is due to substances other than the colored product of urea.

The optical density curves of the products obtained with citrulline and a number of other compounds are indicated in Fig. 2. As already reported (1), citrulline is present in normal blood plasma in concentrations ranging from 0.3 to 1.5 mg. per 100 cc. However, as the concentration of citrulline, weight for weight, is only one-tenth to one-fiftieth that of urea, and as the sensitivity of the method, weight for weight, is 30 times less for citrulline than for urea, the error introduced by citrulline is insignificant, and the removal of citrulline from plasma by adsorbents (1) is unnecessary.

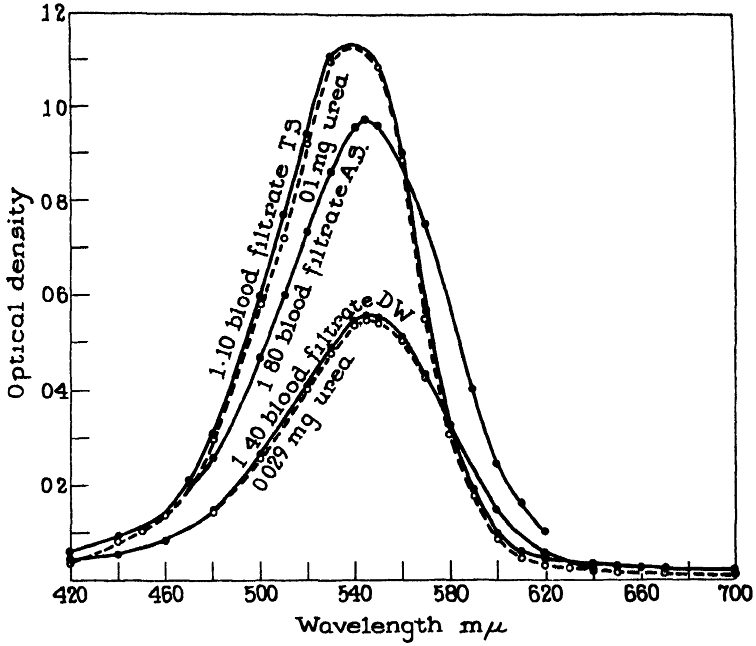

FIG 1 Optical density curves of urea standards; Somogyi filtrates of human blood. 8 cc. of the filtrate were heated 60 minutes at 100° with 5 cc. of sulfuric-phosphoric acid mixture and 0 40 cc. of 4 per cent α-isonitrosopropiophenone. The curves obtained with a blood filtrate of patient T S and the adjacent urea standard were obtained on a Coleman spectrophotometer No. 10-S which has two diffraction gratings. Curves obtained with filtrates of patients A. S. and D. W. and the adjacent urea standard were obtained on a Coleman junior spectrophotometer which has one grating.

Fig. 3 shows the rate of color development in standard solutions heated under the conditions prescribed for analysis.

The optical density developed in a solution when α-isonitrosopropiophenone is heated in acid with different concentrations of urea does not vary in exact proportion to the urea concentration. Fig. 4 shows this clearly. When the optical density is greater than 0.5, calculations based on Beer's law do not hold. (A similar disparity is shown when the color is developed with diacetylmonoxime (1).) When calculations are based on a calibration curve, determinations of blood and urine urea agree well with figures obtained by specific urease methods.

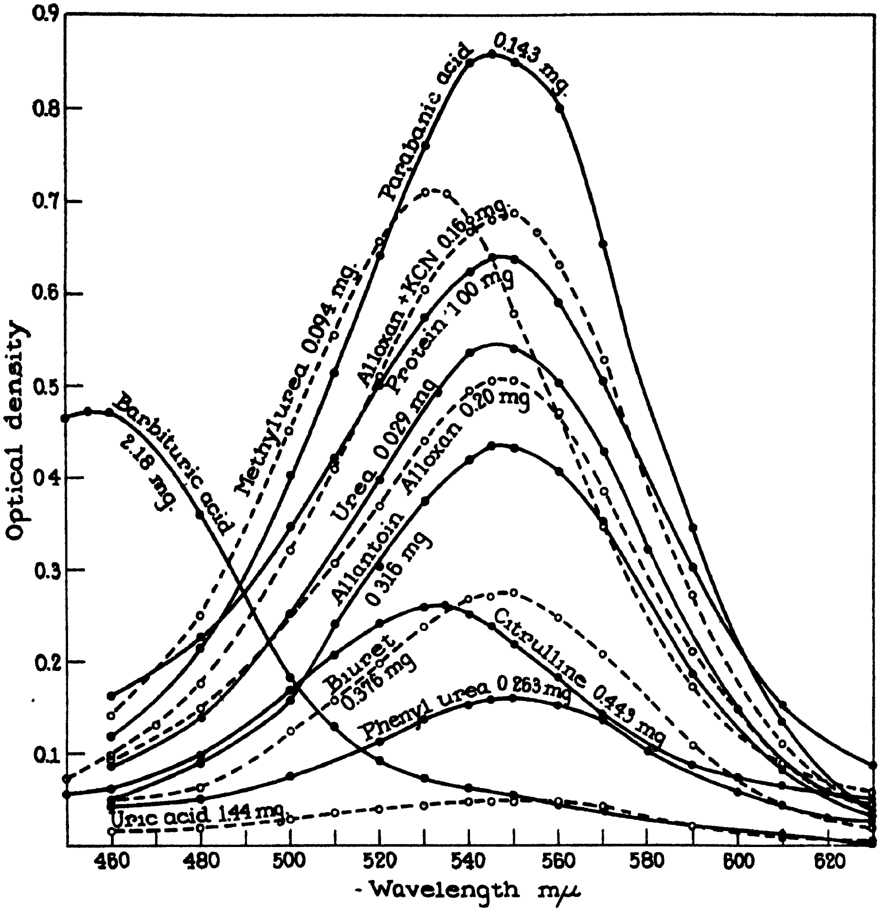

FIG. 2. Optical densities of products obtained with substances giving color on heating in acid with α-isonitrosopropiophenone under the conditions prescribed for measurement of urea. The weights indicate the amount of substance present in 6.20 cc. of the mixture heated 60 minutes at 100° The volume of mixture heated in this case is half that prescribed for routine determination of urea

Table II compares the blood and urine urea levels found by this method with values obtained by the manometric urease method (2, 3).

Specificity of the Method

No color formation results when *ammonia*, *urethane*, *thiourea*, *benzimidazole*, *proline*, *ornithine*, *glutamine*, *asparagine*, *glutamic acid*, *ammonium* *pyrrolidonecarboxylate*, *glutathione*, *ergothioneine*, *caffeine*, *adenine*, *hydantoin*, *acetamide glycocyamine*, or *creatine* is heated under the conditions prescribed for urea determination.

Alloxantin and *uric acid* in relatively high concentrations give a trace of red. *Parabanic acid*, *alloxan*, *alloxan treated with KCN*, *biuret*, *allantoin*, and *protein* give a strong color resembling the red obtained with urea. *Phenylurea* produces relatively little color. *Methylurea* reacts to yield a strong red color with an absorption maximum (525 mμ) lower than that obtained with urea (540 mμ). 10 mg. each of *N-benzyl-N-methylurea*,[3] *N-ethyl-N-o-ethylphenyl-N′-dibenzoylurea*,[3] and *N-ethyl-N-2,4-dimethylphenyl-N′-ethylurea*[3] yield no color with α-isonitrosopropiophenone when heated under the prescribed conditions for 1 hour. Under the same conditions 10 mg. of *N-hydroxyethyl-N-phenylurea*[3] and *N-2-methyl-4-bromophenyl-N′-ethylurea*[3] give only a trace of color, similar in shade to that obtained with urea.

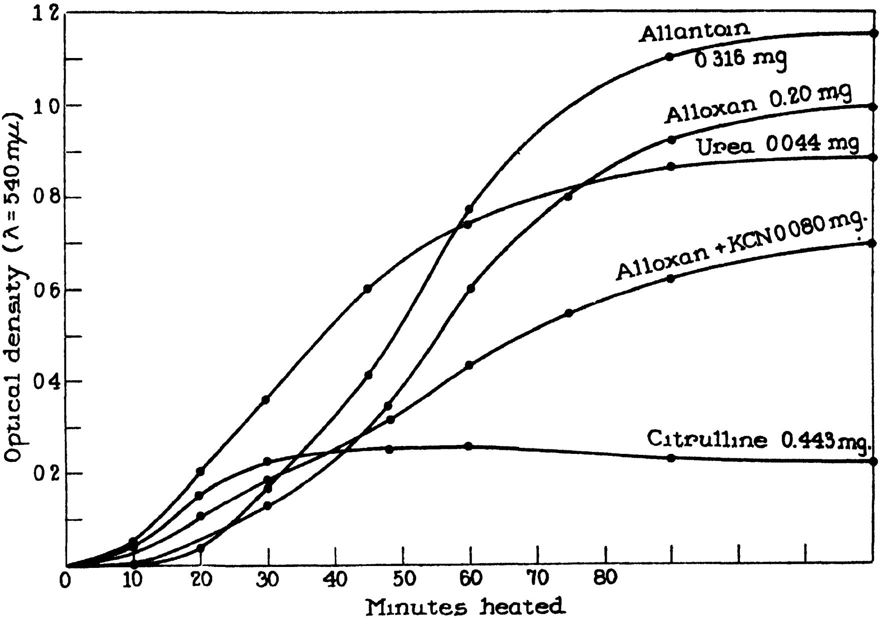

Fig. 3 Rate of formation of color at 100° in reactions of substances with the same concentrations of α-isonitrosopropiophenone and sulfuric-phosphoric acid mixture prescribed for the measurement of urea. The weights indicate the amount of substance heated in the 6.20 cc of mixture This volume of mixture is half that prescribed above for routine determination of urea

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[3] These urea derivatives were obtained through the courtesy of Dr. Richard Baltzly of the Wellcome Research Laboratories, Tuckahoe 7, New York.

Thymol, which in the diacetyl reaction yields a color resembling that obtained with citrulline (1, 11), gives no color with α-isonitrosopropiophenone but causes the development of turbidity if the sample contains more than 0.001 gm

Barbituric acid reacts to give a yellow color with an absorption maximum near 450 mμ. Except with very high concentrations the product causes insignificant absorption at the wave-length used for measuring urea

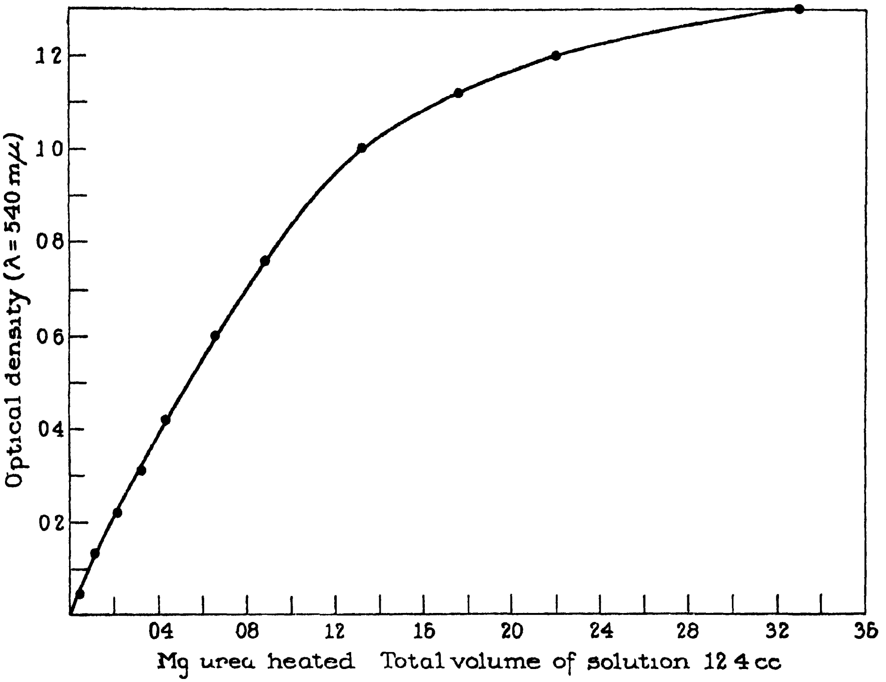

FIG 4. Relation between the optical density at wave length 540 mμ and the amount of urea heated The conditions were as prescribed in the procedure for routine analysis for urea The volume of mixture heated was twice that used in the cases illustrated in Fig 2 or Fig 3

As indicated in a previous communication (1), alloxan is absent from normal dog and human plasma and urine, indeed it is rapidly destroyed in these media This conclusion has been reached by the application of several methods for the determination of alloxan to be published shortly

Nature of Chromogenic Reaction

Although the mechanism of the reaction involved in the formation of color is unknown, some clues as to its nature can be obtained from the study of the structure of the diketo derivatives and the urea derivatives which react. Lang (12) pointed out that, in the case of color formation with guanadine derivatives in alkaline solution, the two carbonyl groups of the diketo compound must be adjacent. The same may be said of the carbamido reaction with α-isonitrosopropiophenone. Benzil, $C_6H_5 \cdot CO \cdot CO \cdot C_6H_5$, does not react to yield a color Lang (12) stated that at least one NH_2 of the guanidine derivative must be free. This is not strictly true in the case of the carbamido reaction. A compound of the structure $R_1NHCONHR_2$ has been listed above as yielding a small amount of color with α-isonitrosopropiophenone. It yields much more color on 10 minutes heating in the diacetyl-carbamido reaction.[4]

TABLE II

Comparison of Whole Blood Urea N, Urine Urea N, and Urea Clearances by Colorimetric and Manometric Urease Methods

Subject	Diagnosis	Whole blood urea N*		Urine urea N*			Urea clearance	
		Colorimetric method†	Manometric urease method	Period	Colorimetric method	Manometric urease method	Colorimetric method	Manometric urease method
		mg per 100 cc	*mg per 100 cc.*		*mg per 100 cc.*	*mg per 100 cc*	*per cent of normal*	*per cent of normal*
G P.	Chronic nephritis	27.8	27.0	1	516	478	42.1	43.0
				2	173	173	43.0	44.0
				3	239	232	37.9	37.0
M. B.	“ “	12.2	11.9	1	1111	1040	133	129
				2	1296	1218	134	131
				3	791	776	108	104
A C	“ “	33.3	33.7	1	310	307	31.1	30.4
				2	269	267	32.5	31.9
C T	Healed “	10.1	9.7	1	152	146	128	128
				2	107	107	148	152
				3	134	118	98	98
A. Si.	“ “			1	668	660		
				2	287	292		
J. A	“ nephrosis	10.2	10.2	1	127	123	91.1	88.0
				2	107	108	65.4	66.0
				3	387	363	85.3	81.0
L R	Latent nephritis	10.9	10.4	1	131	122	158	156
				2	86	86.7	127	134
				3	303	290	128	128
A. St.	“ nephrosis			1	1132	1147		
				2	879	870		

* These figures can be converted to urea by the factor 2.143.

† These values were obtained on Somogyi filtrates.

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[4] R M. Archibald, unpublished results.

Amides with the group $R \cdot CONH_2$ (acetamide, asparagine, glutamine) do not yield color in acid with either diacetylmonoxime or α-isonitrosopropiophenone. Mono-substituted urea derivatives, $R \cdot NHCONH_2$ (methylurea or phenylurea or citrulline), react, giving much more color with diacetylmonoxime than with α-isonitrosopropiophenone. Asymmetrically disubstituted ureas, $R_1R_2NCONH_2$, yield insignificant amounts of color with either reagent. Compounds of the structure $R_1R_2NCONHR_3$ or $R_1R_2NCON(R_3)_2$ yield no color.

As indicated previously (1) diacetyl and its mono- or dioxime yield the same shade of color in the carbamido reaction. The intensity of color obtained, however, is slightly greater when the monoxime is used. Benzoylacetyl and its monoxime, α-isonitrosopropiophenone, likewise yield the same color. It is apparent therefore that the isonitroso group is not essential to the reaction.

Lang (12) observed that benzoylacetyl in alkaline solution could replace diacetyl as a reagent for the determination of guanidine derivatives. Indeed he developed a colorimetric method for creatinine and arginine which involved use of benzoylacetyl As far as the author is aware, this reagent or its monoxime has not been used in acid solution heretofore for the determination of carbamido compounds.

Use of Method for Urea Clearance

Van Slyke and Cope ((13), (3) p. 935) described a colorimetric method for the determimation of urea clearance. This involves dilution of urine to such an extent that, if the clearance is average normal, the urea concentration in the diluted urine equals the concentration in the blood. The urea contents of the diluted urine are then directly compared in a colorimeter; the ratio of the readings indicates the clearance value. This procedure has, over one requiring separate determinations of blood and urine urea contents, two simplifying advantages: (1) only one colorimetric reading is required for each clearance value instead of two, (2) no standard solutions are required. Van Slyke and Cope decomposed this urea with urease and used the nesslerized solutions for colorimetry. The procedure of Van Slyke and Cope is rendered still simpler if the present colorimetric method is used.

Accuracy of the Method

There is present in human urine and blood material other than urea, which reacts with α-isonitrosopropiophenone (and to an even greater extent with diacetyl). Probably part of this material is allantoin.

In blood the amount of this material is slight compared with the amount of urea. It is only enough to raise the apparent urea nitrogen determined by the colorimetric method by 0.0 to 0.8 mg. per 100 cc. above the value determined with urease, as exemplified by the results in Table II.

Urine urea values (Table II) obtained by the colorimetric method average 2.8 per cent higher than those obtained by the manometric urease method. The standard deviation from this mean difference for twenty-two specimens (eight patients) is ±3.0 per cent. Because urea clearance values are calculated from the ratio of the urea concentration in blood to that in urine, the small positive errors in the concentrations determined colorimetrically in urine and blood approximately compensate each other. The positive errors in the urine are slightly larger than in the corresponding bloods, and urea clearances determined colorimetrically (Table II) average 0.8 per cent higher than those determined by the gasometric urease method. The standard deviation from this mean difference is ±2.6 per cent. The agreement with the clearances determined by the manometric urease method is somewhat better than that obtained by Van Slyke and Cope (13) using colorimetry based on nesslerization.

The colorimetric method at present cannot be recommended for the determination of urea when a high degree of accuracy is required. However, the magnitude of the error in the colorimetric determination of urine urea is usually much smaller than that involved in the measurement of the rate of urine flow. Incomplete emptying of the bladder during urea clearance tests, especially with small urine flow, leads to errors much larger than those involved in measurement of urea concentration. Therefore the method, as it stands, will be sufficiently accurate for most clinical purposes.

It is hoped that at some future date there will be opportunity to investigate more extensively the cause, frequency, and degree of deviation of the colorimetric urea results so that a modification of the method may be used for the accurate determination of urea.

The author is indebted to Dr F. C. Uhle for the suggestion that α-isonitrosopropiophenone might be a less volatile substitute for diacetylmonoxime in the colorimetric determination of urea.

SUMMARY

1. A simple colorimetric method for the determination of urea has been outlined. It is based on the red color formed when urea is heated in acid with α-isonitrosopropiophenone.

2. Neither ammonia nor products ordinarily encountered in human blood filtrates interfere appreciably with the analysis.

3. When applied to urine, the method gives urea values averaging 2.8 per cent higher than the concentrations indicated by the urease method. Hence application to urine is limited to cases in which approximate values suffice; it is not recommended for exact determination of the nitrogen distribution among urinary constituents.

4. The method lends itself well to determination of urea clearances by

direct colorimetric comparison of diluted urine with blood filtrate, as described by Van Slyke and Cope. It is more convenient than the nesslerization procedure which these authors used, and yields results agreeing more closely with those obtained by the exact manometric urease method.

BIBLIOGRAPHY

1. Archibald, R. M , *J. Biol. Chem.*, **156**, 121 (1944).
2. Van Slyke, D. D., *J. Biol. Chem.*, **73**, 697 (1927).
3. Peters, J. P., and Van Slyke, D. D , Quantitative clinical chemistry, Methods, Baltimore, 372 (1932, 1943).
4. Van Slyke, D. D., and Hawkins, J. A., *J. Biol. Chem.*, **79**, 742 (1928).
5. Somogyi, M., *J. Biol. Chem* , **87**, 339 (1930).
6. Fujita, A., and Iwatake, D., *Biochem. Z.*, **242**, 43 (1931).
7. Miller, B. F., and Van Slyke, D. D., *J. Biol. Chem.*, **114**, 583 (1936).
8. Folin, O., and Wu, H., *J. Biol Chem.*, **38**, 81 (1919).
9. Hamilton, P. B., and Archibald, R. M , *Ind. and Eng. Chem* , *Anal. Ed.*, **16**, 136 (1944).
10. McIntosh, J. F., Møller, E , and Van Slyke, D. D., *J. Clin Invest.*, **6**, 467 (1928).
11. Barker, S. B., *J. Biol. Chem.*, **152**, 453 (1944).
12. Lang, K., *Z. physiol. Chem.*, **208**, 273 (1932)
13. Van Slyke, D. D., and Cope, C L , *Proc Soc Exp. Biol. and Med* , **29**, 1169 (1932).

THE SYNTHESIS OF UREA FROM GLUTAMINE BY LIVER EXTRACT

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(Received for publication, November 9, 1944)

Leuthardt and Glasson (1, 2), Krebs (3), and Bach (4) have shown that slices of fasting guinea pig liver convert glutamine (and to a much smaller extent asparagine and glutamic acid) to urea. The present report shows that the enzyme responsible for this conversion can be extracted from liver cells of guinea pig, beef, dog, and human, dialyzed, and separated from insoluble débris, that it is relatively heat-stable, is not inhibited by bromosulfalein, is markedly inhibited by cyanide, and is active after precipitation from aqueous solution by an equal volume of acetone. Either the system is reversible or another system is present which removes urea as it is formed. The concentration of urea reaches a maximum long before all the glutamine is utilized.

Method of Preparation of Extract

Liver, either fresh or stored frozen on solid CO_2, is sliced, placed in a Waring blendor with 3 volumes of water and 1 gm. of $MnCl_2 \cdot 4H_2O$ per 10 gm. of tissue, and homogenized for 10 minutes. It is then heated to 50° for 20 minutes and centrifuged.

The clear supernatant is then dialyzed for 2 hours in sausage casing against 100 volumes of distilled water in a shaking machine. This serves to remove preformed urea, glutamine[1] and arginine, citrulline and ornithine, thereby reducing the enzyme blank. The preparation at this stage may be stored frozen in solid CO_2, or as a powder after drying from the frozen state.

Alternatively, after heating the mixture to 50° the supernatant may be cooled to 0° and treated in the cold room with ⅓ volume of cold acetone. The precipitate obtained is centrifuged off and discarded. It contains only an insignificant portion of the activity. The supernatant is treated with half its volume of cold acetone and the precipitate obtained on cold centrifugation is washed with acetone and dried in a vacuum desiccator. The supernatant, which contains among other things the substances which lead to high blanks, is inactive and is discarded.

Discussion of Method of Preparation of Extract

Heating to 50° inactivates a number of enzymes, including glutaminase, and precipitates many proteins without decreasing the activity of the preparation. The addition of $MnCl_2$ is not essential but aids in removing inactive protein. Its use neither increases nor decreases the activity of the preparation. As has been shown by the author[2] (5), such treatment causes activation of the arginase present.

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[1] Hamilton, P. B., unpublished work.

Method of Testing Activity of Extracts

To tubes containing 1 cc. of 1 per cent solution of glutamine and 1.0 cc. of 0.5 M sodium phosphate buffer of pH 7.10 are added 0.5 cc. portions of the 25 per cent extracts to be tested. In control tubes 1 cc. of water replaces the 1 cc. of glutamine solution. After 30 minutes incubation at 40° the tubes are placed for 4 minutes in a boiling water bath to inactivate the enzyme, then centrifuged. 2 cc. aliquots of the supernatants are dialyzed for 2 hours against 10 cc. of water in the dialysis units described by Hamilton and Archibald (6).

Urea is determined colorimetrically according to the technique of Archibald (7). 4 cc. aliquots of dialysate are heated in a boiling water bath for 1 hour with 2 cc. of phosphoric-sulfuric acid mixture[3] and 0.2 cc. of 4 per cent α-isonitrosopropiophenone in alcohol. Appropriate standards and reagent blanks are heated at the same time.

The specificity of the enzyme reaction has been checked by treating the dialysates with enough dialyzed (8) urease to destroy urea. Such treatment decreases the color obtained in dialysates of liver extract digests to that found in the reagent blank.

Discussion of Results

The amounts of urea formed when different substrates and inhibitors and sources of enzyme are used are indicated in Table I. It will be observed that the enzyme is not adherent to the insoluble cellular débris, since it is slightly more concentrated in the clear solution than in the whole mash. It is present in dog, beef, guinea pig, and human liver. Extracts of dog, beef, and human liver in 25 per cent glycerol heated to 50° according to the directions of Hunter and Dauphinee (9) for preparation of arginase, after storage for 4 years at 4°, contain, besides arginase, considerable and perhaps nearly all of the original activity of the enzyme systems responsible for synthesis and removal of urea. It is not activated by addition of any of the

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[2] Van Slyke, D. D., Archibald, R. M., and Rieben, W. K., unpublished data. Hunter and Downs quote unpublished work of R M Archibald

[3] To maintain the same concentration of sulfuric and phosphoric acids and of α-isonitrosopropiophenone as is prescribed for the routine determination of urea (7), the acid mixture is prepared by mixing 1 volume of sulfuric acid with 3 volumes of phosphoric acid (without the addition of 1 volume of water)

TABLE I

Urea Formed from Glutamine by Extract of Liver in 30 Minutes at 40°

Substrate, 1 cc	Liver extract from	Molar phosphate buffer, 1 cc	Enzyme preparation Concentration, 1 part liver, 3 parts water 0.5 cc	Added	Urea in 2.5 cc digest
		pH			*mg*
Glutamine 1%	Dog	7.1	Liver mash unheated dialyzed (whole)		0.013
			Liver mash unheated dialyzed (supernatant)		0.025
			Liver mash unheated dialyzed (whole)	Bromosulfalein 100 mg.	0.040
			Liver mash heated to 50° (supernatant)		0.047
			“ “	Bromosulfalein 100 mg.	0.059
			“ “	KCN to give 0.008 M	0.006
			Liver mash heated to 60° (supernatant)		0.066
			Liver mash heated to 70° (supernatant)		0.007
			Liver mash heated to 100° (supernatant)		<0.002
			Liver mash heated to 50° with $MnCl_2$ (supernatant)		0.051
Glutamine 1%	Human 1	7.1	Liver mash heated to 50° (supernatant)		0.037
	2				0.045
	3				0.040
Glutamine 1%	Beef	7.1	“ “		0.014
Glutamine 1%	Guinea pig	6.50	Liver mash unheated dialyzed (whole)		0.052
		7.06	“ “		0.044
		7.40	“ “		0.028
Glutamic acid 1% and NaOH to pH 7.1	Dog	7.1	Liver mash heated to 50° dialyzed (supernatant)		0.016
Asparagine 1%			“ “		0.027

materials tested but is inhibited by KCN.[4] The apparent increased activity resulting from heating whole liver mash, or from addition of bromosulfalein to unheated liver, is due presumably to the resulting inactivation of glutaminase. Bromosulfalein, which inhibits glutaminase (10), does not alter the activity of preparations which have been heated to 50° or more. The production of urea under conditions in which glutamine hydrolase is inactive is indication of the validity of the contention of Leuthardt (11) that urea production from glutamine does not involve preliminary hydrolysis of the amide group and subsequent synthesis from ammonia and carbon dioxide.

The pH optimum for the reaction is about 7.0 or less, in contrast to the optimum of arginase, which is about 9.4. This fact, as well as the fact that manganese treatment, which causes a 2- to 3-fold activation of arginase, did not increase the concentration of urea attained, suggests that arginase is either not involved in the reaction, or at least that its activity is not a limiting factor. Further, the use of borate buffer (which markedly inhibits arginase) does not inhibit urea formation by this enzyme system. Since cell-free extract of liver cannot convert citrulline to arginine, it is unlikely that Krebs' ornithine cycle (12) is involved in the reaction studied here. Ornithine, phenylacetic acid, and penicillin have no effect on the activity of the preparation or the concentration of urea achieved.

Leuthardt and Glasson (2) report that vitamin B_1 and its phosphate ester catalyze urea synthesis in vitamin B_1-deficient rats. We have been unable to note any influence of either vitamin B_1 or bicarbonate buffer on the equilibrium reached in this enzyme system *in vitro*.

No urea is formed when glutamine is replaced by *alanine*, *ornithine*, *proline*, *ammonium pyrrolidonecarboxylate*, *glycine*, *adenine*, *ammonium pyruvate*, or *ammonium chloride*.

The concentration of urea formed by cell-free extract of liver reaches a maximum at 38° in about 15 minutes. The concentration of urea achieved decreases with decreased concentration of glutamine, but a 20-fold change in the concentration of the enzyme is almost without effect on the urea concentrations attained. If urea is *added* to the glutamine-liver extract digest to give a concentration greater than that present in the equilibrium mixture, the concentration of urea achieved during incubation is less than the sum of the amount added and the maximum ordinarily reached. This leads one to conclude either that the system is reversible or that at least two separate reactions are involved, one of which causes synthesis of urea from glutamine; the other removes urea from the system. Work is under way which will, it is hoped, lead to identification of the other products of the reaction. At present it seems appropriate that assignment of names to the enzyme systems responsible should wait until the nature of the products (other than urea) has been established.

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[4] Although cyanide inhibits the reaction, urea formation from glutamine does not require molecular oxygen. The same concentration of urea is reached when the oxygen in the system is displaced with water vapor by evacuation as when air is present at atmospheric pressure.

Ammonia does not accumulate in the system to an extent greater than is to be expected from the spontaneous breakdown of glutamine in phosphate buffer.[1] Ammonia is not the product resulting from the disappearance of urea from the system.

The presence of these enzyme systems in arginase preparations leads one to consider again the specificity of the enzymatic determination of arginine in enzymatic or acid hydrolysates of protein containing glutamine and asparagine or the corresponding dicarboxylic acids. If during incubation with the arginase preparation urea is synthesized from sources other than arginine, results would indicate too high a concentration of arginine. If, on the other hand, urea is removed by the system, results would appear too low. Fortunately, however, in such analysis arginase usually acts at a pH well above the optimum for urea synthesis from glutamine. Also, in the products of acid hydrolysis of proteins, no glutamine is present, since the conditions of the hydrolysis convert it quickly into glutamic acid and ammonia.

SUMMARY

1. Cell-free extracts of beef, dog, guinea pig, and human liver form urea from glutamine.

2. At 40° urea concentration reaches a maximum in 15 minutes, which corresponds to a transformation of 3 per cent of the amide N or amino N of glutamine to urea nitrogen.

3. Formation or accumulation of urea is prevented by the presence of cyanide, but is not affected by absence of molecular oxygen.

4. Ammonia is not a product of the reaction involving removal of urea from the system. Formation of urea from glutamine does not involve hydrolysis of the amide link by glutaminase.

BIBLIOGRAPHY

1. Leuthardt, F., and Glasson, B., *Helv. chim acta*, **25**, 630 (1942). Leuthardt, F., *Z. physiol. Chem.*, **252**, 238 (1938).
2. Leuthardt, F., and Glasson, B., *Helv physiol. acta*, **1**, 221 (1943)
3. Krebs, H. A., *Biochem. J.*, **36**, 758 (1942)
4. Bach, S. J., *Biochem. J* , **33**, 1833 (1939).
5. Hunter, A., and Downs, C E., *J Biol. Chem.*, **155**, 173 (1944).
6. Hamilton, P. B , and Archibald, R. M , *Ind and Eng. Chem., Anal. Ed.*, **16**, 136 (1944).
7. Archibald, R. M., *J. Biol. Chem* , **157**, 507 (1945).
8. Archibald, R. M., and Hamilton, P. B , *J. Biol Chem* , **150**, 155 (1943).
9. Hunter, A., and Dauphinee, J., *J. Biol. Chem.*, **85**, 627 (1929–30).
10. Archibald, R. M., *J. Biol. Chem.*, **154**, 657 (1944).
11. Leuthardt, F., *Z. physiol. Chem.*, **265**, 1 (1940).
12. Krebs, H A., and Henseleit, K., *Z physiol Chem* , **210**, 33 (1932).

BLOOD ALLOXAN AND BLOOD GLUTATHIONE IN RABBITS INJECTED WITH ALLOXAN*

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Although the production of diabetes (1–5) in animals as a result of alloxan injections is now an established fact, little information is available pertaining to the biological activity of the drug. Obviously, any means of revealing the course of the injected alloxan will be of value in understanding the mechanism of its action. Two procedures have been developed in this laboratory which may be used in determining blood alloxan. Although neither of these gives a specific test for alloxan, we feel that the procedures have enabled us to follow blood levels of alloxan in injected animals with considerable accuracy. The methods with the results of their application, together with a study of some biochemical reactions following the injection of alloxan, comprise the basis of this paper.

In 1932 Lieben and Edel (6) used the reaction between ammonium sulfide and alloxan in a quantitative colorimetric estimation of alloxan. The color produced by ammonium sulfide is very unstable and the proportionality between color and concentration of alloxan holds only over extremely limited ranges. In our hands it did not prove satisfactory. This procedure is the only report on a quantitative method for measuring alloxan which we have been able to find in the literature.

Determination of Alloxan. Ferricyanide Method.—We have found that alloxan may be determined quantitatively through its ability to reduce ferricyanide in the cold, using a modification of the Folin micromethod (7, 8) for blood sugar. This reaction can, under properly controlled conditions, be used in determining alloxan in tungstic acid blood filtrates in amounts varying between 0.05 and 1 mg.

Before outlining the procedure used for the determination as applied to blood containing alloxan from injected animals, a brief discussion of preliminary work may be helpful.

First, if a known amount, 0.1 mg. to 0.4 mg., of alloxan is added to a

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* We are deeply indebted to Dr. H. C. Trimble of the Department of Biological Chemistry, Harvard Medical School, for his valuable advice and assistance in this work. We are indebted for technical assistance to Miss Laura DuBreuil, Mrs. L. T. McDaniel, and Miss Louise Sheldon.

To the Lederle Laboratories, Inc., of the American Cyanamid Company we are indebted for a grant in support of this investigation. The alloxan used was supplied by the American Cyanamid Company.

123. From these results it is evident that alloxan does not affect the copper method, while it obviously reduces the ferricyanide in the Folin method.

It would appear, therefore, that a copper method for blood sugar is definitely superior to a ferricyanide method in alloxan work. However, as will be shown subsequently, it is only in those bloods taken immediately after injection that there may be a significant positive error in accepting the observed blood sugar values based on the Folin micromethod as the actual blood sugar concentration. With careful technique one may gain an essentially true picture of blood alloxan at this point by determining blood sugar by the two methods, and converting the difference obtained to alloxan. Some illustrations of this procedure are presented in Table III.

Recovery of Alloxan Added to Human Plasma—As already shown, little difficulty is encountered in recovering alloxan which has been added to any tungstic acid filtrate. When alloxan is added to whole blood, serum, or plasma, however, there appears to be a rapid destruction of the alloxan as measured by its reducing ability. 0.2 cc. of 10 per cent alloxan was added to 2 cc. of fresh plasma and placed in an incubator at 37°. At intervals 0.1 cc. samples were withdrawn, deproteinized with tungstic acid, and their reducing ability determined by applying the ferricyanide procedure to the filtrate. The reducing action diminished rapidly (Fig. 1) and almost completely disappeared in 8 minutes. The probability that at least a part of this destruction is due merely to the alkalinizing factors of blood plasma is suggested by the marked effect an increase in pH has on the reducing properties of an alloxan solution. In one series of experiments alloxan was maintained at room temperature with buffers of pH 6.5 to 8.0. At intervals 4 cc. aliquots were withdrawn and the reducing ability was determined by

TABLE III

Alloxan in Blood Calculated from Blood Sugar Values Obtained by Ferricyanide Method and Copper Method

Rabbit No	Blood sugar, Folin ferricyanide method	Blood sugar, copper method	Difference	Blood sugar difference as alloxan*	Alloxan determined by ferricyanide method†
	mg per 100 cc	*mg per 100 cc*		*mg per 100 cc*	*mg per 100 cc*
77	127	107	20	133	130
114	155	117	38	253	262
114	137	126	11	73	66
115	108	93	15	100	100

* Calculated according to formula in text, blood sugar difference × 6.67 = blood alloxan.

† Present authors' ferricyanide method (see the text).

our ferricyanide procedure. The reducing ability steadily diminished with time. The results are shown in Fig. 2. Similar results were obtained when alloxan was partly or wholly neutralized with NaOH (Fig. 3). Since alloxan in phosphate buffers on the acid side of neutrality tends to show a greater reducing ability than alloxan in water, the initial values of the 6.5 and the 7.0 curve in Fig. 2 exceed 100 per cent. These experiments corroborate the findings of Labes and Freisburger (9) who observed rapid destruction of alloxan under some conditions.

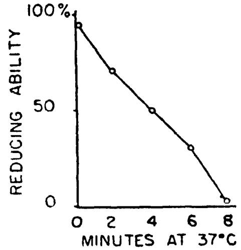

FIG 1. Loss in reducing ability of alloxan in fresh human plasma The data represent per cent of original value

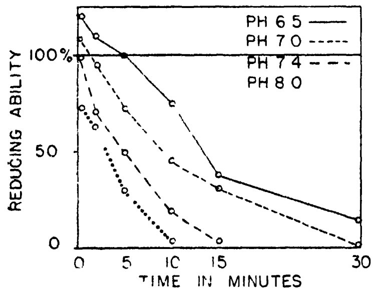

FIG 2

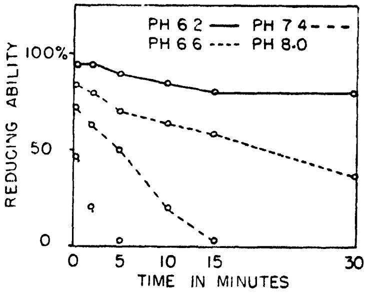

FIG 3

FIG 2 Loss in reducing ability of alloxan in Sorensen's M/15 phosphate buffers during 30 minutes at room temperature Zero minute represents aliquots withdrawn immediately after complete mixing

FIG. 3 Loss in reducing ability of alloxan in solution partially or wholly neutralized with NaOH during 30 minutes at room temperature

Ferricyanide Method for Determination of Blood Alloxan

Reagents—

1. A fresh, accurately prepared strong tungstic acid solution. Introduce 16 cc. of 10 per cent sodium tungstate into 50 to 60 cc. of water in a 100 cc. volumetric flask. Add 16 cc. of $\frac{2}{3}$ N sulfuric acid, mix, and dilute to 100 cc.

with distilled water. This solution is 8 times the strength of the Folin dilute tungstic acid (7).

2. 0.4 per cent potassium ferricyanide solution prepared from the purified salt according to Folin (7).

3. Sodium carbonate-sodium cyanide solution prepared according to Folin (7) as follows. To 8 gm. of anhydrous sodium carbonate dissolved in about 200 cc. of distilled water in a 500 cc. volumetric flask add 1.5 gm. of sodium cyanide dissolved in 150 cc. of distilled water. Mix and dilute to 500 cc.

4. Ferric iron-gum ghatti solution adapted from Folin (8). Place 20 gm. of gum ghatti in an open top bag made of several thicknesses of cheesecloth. Immerse the bag in a liter cylinder filled with water, attaching the top of the bag to the top of the cylinder by means of a rubber band. Allow to stand overnight. Discard bag and contents. To the gum ghatti solution in the cylinder add a solution of ferric sulfate prepared as follows. Dissolve with the aid of heat in a 250 cc. beaker 5 gm. of anhydrous ferric sulfate, 75 cc. of 85 per cent phosphoric acid, and 100 cc of water. After mixing the gum ghatti solution with the cooled ferric sulfate solution add, a few cc. at a time, 15 to 20 cc. of 1 per cent potassium permanganate. Mix after each addition. When sufficient permanganate has been added, the pink color produced by its addition will disappear slowly.

5. Alloxan standard. (*a*) Stock standard: Weigh accurately 100 mg. of alloxan monohydrate and dissolve in 100 cc of distilled water. This stock solution will keep at least 1 week in the refrigerator if the original alloxan is pure white in color. (*b*) Dilute standard: A 1:10 dilution in distilled water of the stock. The dilute standard contains 0.1 mg. per cc. This dilute standard should be prepared fresh each day.

Standardization of Alloxan Standard—Although we have found little variation in the reducing ability of various samples of alloxan when determined under the same conditions, we believe standardization of the alloxan against the regular micro blood sugar standard is desirable. To do this, pipette exactly 4 cc. of the dilute alloxan standard into a micro blood sugar tube. Add 1 cc. of ferricyanide followed by 1 cc. of cyanide carbonate. Mix by rotation and allow to stand 2 minutes at room temperature. Add 5 cc. of ferric sulfate-gum ghatti solution, distilled water to 25 cc., mix, and read against a Folin micro blood sugar standard heated in the usual way. Since the heating factor may vary it is well to standardize the alloxan against two or three blood sugar standards heated at different times and to average the results obtained. We have found 0.4 mg. of alloxan in water solution invariably to give a blood sugar equivalent of 115 to 120, usually about 120. Since a blood sugar of 120 mg. per 100 cc. represents 0.048 mg. of glucose, it follows that alloxan in water has 12 per cent of the reducing strength of glucose.

Alloxan in a strong tungstic acid solution shows a greater reducing ability, becoming 15 per cent that of glucose. The ratio between the reducing strength of alloxan in water and in strong tungstic acid averages 0.81. Therefore, when alloxan in a strong tungstic acid filtrate is read against a water standard of alloxan, it is necessary to multiply the result by 0.81. Although an alloxan solution in tungstic acid may be used as a standard, it is not so constant as a water standard and, furthermore, shows a marked tendency to break down the Prussian blue colloid. We prefer either the dilute water standard of alloxan or the regular micro blood sugar standard.

Procedure—Introduce 0.5 cc. of oxalated blood directly into 9.5 cc. of strong tungstic acid. Stopper and shake hard immediately. Centrifuge and decant the supernatant fluid. (Important: Not more than a few seconds should elapse between the withdrawal of blood from the animal and the precipitation in tungstic acid. It is our custom to withdraw 2 to 3 cc. of blood from the rabbit's heart in a syringe containing oxalate, eject it immediately into an oxalated tube, and pipette directly 0.5 cc. into the tungstic acid solution With skill this entire procedure can be accomplished in 30 seconds.) The Folin micro blood sugar procedure is then applied to the filtrate as follows.

Pipette exactly 4 cc. of filtrate into a blood sugar tube graduated at 12.5 and 25 cc. Add *in the order given* 1 cc. of 0.4 per cent potassium ferricyanide and 1 cc. of cyanide-carbonate solution. Mix by rotation and let stand 2 minutes at *room temperature* in a *dark corner*. Add 5 cc. of ferric sulfate-gum ghatti solution and make to volume, either 12.5 cc. or 25 cc., depending on the color obtained. Keep the tubes away from strong light and compare within 5 to 10 minutes in a Duboscq colorimeter with an alloxan standard containing 0 2 or 0.4 mg. of alloxan, or a heated micro blood sugar standard which has been prepared simultaneously. Under these conditions the development of color in the blank is usually not sufficient to cause serious error As before stated, alloxan reduces ferricyanide almost immediately and full color development is obtained within 5 minutes after the addition of the ferric salt. If the tubes are allowed to stand in the light or if the color comparison is not made within the specified time, there may be a large positive error in tubes containing small amounts of alloxan.

Calculation—If the determination is made by the ferricyanide procedure of the present authors and the color read against an alloxan standard, the calculation is

$$\frac{\text{Reading of standard}}{\text{Reading of unknown}} \times 0.4 \times 0.81 \times 500 = \text{mg. alloxan per 100 cc. blood}^{2}$$

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[2] 0.81 is the factor used because the standard is in water, while the unknown is in tungstic acid.

On the other hand, if the color of the solution containing alloxan is measured against a heated sugar standard, the calculation is

$$\frac{\text{Reading of standard}}{\text{Reading of unknown}} \times 0.04 \times 6.67 \times 500 = \text{mg alloxan per 100 cc. blood}$$ [3]

Cyanide-Carbonate Color Test for Detection of Alloxan in Tungstic Acid Blood Filtrates—When appreciable amounts of alloxan are present in the blood, the ferricyanide procedure described affords a satisfactory method for its determination. When only traces of alloxan are present, however, the color obtained is frequently difficult to interpret, since color changes occur, due to the reagents or to other factors which may be present in the blood. In these bloods, containing only traces of alloxan, it is helpful to apply a second test which, though only qualitative at the present time, is apparently quite specific for alloxan in blood.

The qualitative test is as follows: To 4 cc. of strong tungstic acid filtrate prepared as previously directed add either 1 or 2 cc. of the cyanide-carbonate solution of Folin used in the micro blood sugar procedure. *Caution. These two solutions should be added together in the hood.* A blue color develops instantly in the presence of alloxan, owing presumably to the reduction of the tungstic acid.

The only normal blood constituent which we have found which reacts positively with this test is ascorbic acid and it apparently does not cause any interference under the conditions recommended. This test has been applied to a considerable number of bloods, normal and diabetic human bloods and many different rabbit bloods, and we have never had a positive result except in blood known to contain alloxan. The blank is always a water-clear colorless solution. Although conditions have not yet been perfected for stabilizing the color produced with weak alloxan solutions for a quantitative determination, the color produced is proportional to the amount of alloxan present. As little as 0.02 mg. of alloxan gives a definite blue color, and 0.05 mg. will give a sufficient color for a colorimetric reading if fading can be prevented. The color obtained should be observed immediately upon addition of the reagent, as in weak solutions it is transitory. Any blue color which develops over a period of time due to evaporation should be discarded. Further work on this test is in progress.

Blood Alloxan in Rabbits—In all of the animal work to be described Dutch or chinchilla male rabbits 3 to 4 months old have been used. Unless otherwise specified, the alloxan has been given by intravenous injection usually into the marginal vein in the ear. No anesthesia has been used. A freshly prepared 5 per cent solution of alloxan has been used throughout.

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[3] 0.04 = mg. of glucose in the sugar standard. The factor 6.67 is the factor for converting alloxan data, expressed as mg. of blood sugar, to alloxan expressed as such. It is based on the fact that the reducing ability of alloxan in strong tungstic acid is 15 per cent that of glucose.

We have followed blood alloxan levels in a series of twelve rabbits receiving 200 mg. per kilo of alloxan, with both the ferricyanide quantitative procedure and the cyanide-carbonate qualitative test for alloxan (Table IV). Since at the present time the cyanide-carbonate test is not quantitative, we have indicated the color obtained qualitatively by this test with plus signs.

TABLE IV

Blood Alloxan and Blood Reduced Glutathione in Rabbits after Injection of Alloxan, 200 Mg. per Kilo

Experiment No	Rabbit No	Total alloxan injected	Blood alloxan, mg per 100 cc (Control, 0 throughout)				Rabbit No	Blood glutathione, mg per 100 cc					Greatest per cent drop
			After injection						After injection				
			0 min	1–2 min	5 min	10 min		Control	0 min	1–2 min	5 min	10 min	
		mg											
1	69	458	+++	+	0	0							
2	70	322	+++	Died									
3	71	592	67	+	0	0	71	46.9	20.2	21.6	22.7	21.8	57
4	77	345	130	0	0		77	38.6	0	5 (Approximate)	28.6		100
5	114	243	262*	66	0		114	28.9	0	14.5	22.8		100
6	115	254	280*	100	0		115	31.9	0	5 (Approximate)	18.7		100
7	116	277		+	0	0	116	60.0		23.4	23.0		61
8	120	291	+	0	0		120	49.9	25.8			30.0	48
9	76	255	294*	Died									
10	81	310	20	+	Slight +	0							
11	82	370	306*	30									
12	83	347	41		Slight +	0							

* Since insufficient time has elapsed at this point for a uniform distribution of alloxan in the blood, the bloods taken are not truly representative samples and therefore results calculated per 100 cc. may give results higher than the injected amount.

In view of the preliminary work described, it is not surprising that our experiments in alloxan recovery all support the hypothesis that alloxan injected intravenously into rabbits is destroyed or removed from the blood stream very rapidly. The *in vitro* destruction of alloxan in blood plasma as shown in Fig. 1 appears to be duplicated *in vivo*. Table IV shows clearly that in order to recover any large proportion of the injected alloxan, blood must be removed from the animal simultaneously with the injection. The technique which has proved most successful with us has been for one person to withdraw blood from the heart while another injects the last portion of the alloxan solution. A third person precipitates the blood immediately upon its withdrawal. From our experience we cannot emphasize too strongly the necessity of speed if alloxan, as determined by its reducing ability, is to be recovered after injection.

An analysis of the results shown in Table IV indicates that the highest concentration of alloxan in the blood of rabbits injected with 200 mg. per kilo occurs at the end of the injection and is followed by a rapid fall. Three of six rabbits 1 to 2 minutes later show only one-tenth to one-third the value found at the completion of the injection, and only two out of eight rabbits show as much as a trace of alloxan in the blood 5 minutes after its administration. In Rabbits 70 and 76, both of which died immediately at the end of the injection, blood was taken during the injection period as well as at its completion. Positive tests were obtained in all the bloods, the concentration increasing markedly up to the period at the end of the injection.

In all of our experiments on alloxan recovery we have used a 5 per cent solution of alloxan and the injection has been made fairly rapidly, varying from 2 to 4 minutes save in Rabbit 70, which took 7 minutes. In four animals, not recorded in the paper, when difficulty was encountered in making the complete injection rapidly at one time, no appreciable amount of alloxan was recovered. We feel certain that speed of injection, concentration of alloxan solution, and rapid precipitation of the blood all are important factors in alloxan recovery.

Blood Alloxan and Blood Reduced Glutathione—Although our early experiments in alloxan recovery were entirely unsuccessful, an analysis of the results in these experiments suggested the possibility that a part of the immediate destruction of alloxan was brought about by glutathione. The apparently simultaneous disappearance of alloxan and of non-glucose reducing substances in the blood, as found in our experiments with the two blood sugar methods, indicated such an interaction. Labes and Freisburger (9) have suggested the possibility of alloxan reacting with sulfhydryl groups. It seemed of interest, therefore, to determine blood glutathione levels in rabbits injected with alloxan.

The Benedict-Gottschall method (10) as modified by Potter (11) seemed best adapted for frequent determinations on small samples of blood. We found that satisfactory analyses can be made by this procedure with 0.7 cc. of blood which can easily be obtained from the ear vein of a rabbit. By rapid centrifugation of the protein precipitate and filtration of the supernatant fluid through a small plug of washed cotton, 4 cc. of filtrate can easily be obtained, which is sufficient to give good colorimetric readings.

Eastman Kodak glutathione has been used in making the standards. We have used both a standard prepared in HCl as directed in the Benedict procedure and a standard according to Schelling (12) in H_2SO_4. The standards check each other and have given essentially the theoretical titer with iodate.

Changes of reduced glutathione in blood have been followed *first* in conjunction with blood alloxan determinations in six rabbits receiving 200 mg. of alloxan per kilo, *second* in a large series of rabbits receiving one single injection of alloxan varying from 20 mg. per kilo to 200 mg. per kilo, and *third* in four rabbits over a period of days during the development of diabetes through repeated injections of small doses of alloxan.

In the first series of rabbits, Nos. 71, 77, 114, 115, 116, and 120 in Table IV, there is indication of an immediate interaction, the reduced glutathione falling to zero or near zero in three out of five cases almost simultaneously with the disappearance of alloxan The alloxan determinations and the glutathione determinations were made on the same sample of blood withdrawn from the heart in the manner previously described. For both determinations the blood was precipitated as quickly as possible after its withdrawal. The disappearance of alloxan paralleling the marked decrease in reduced glutathione substantiates the hypothesis of an immediate action of alloxan with glutathione through its sulfhydryl group.

In the second series of rabbits, data in Table V, blood was taken from an ear vein at 10 and 30 minute intervals following the alloxan injection and between the 24 and 48 hour period subsequently. Blood reduced glutathione changes and blood sugar levels were followed to determine whether any relationship existed between the dose of alloxan, blood glutathione changes, and development of diabetes. The results in this second series indicate a tendency for an immediate and marked lowering in blood reduced glutathione following the injection of alloxan in doses above 80 mg. per kilo. Since in this series no bloods were taken before the 10 minute period, the low glutathione figure recorded may not always represent actually the lowest point. The glutathione changes, in general, parallel the amount of alloxan injected. With small doses, under 80 mg. per kilo, only slight changes if any occur at the 10 minute period. Above 80 mg. per kilo a marked tendency is observed for the 10 minute postinjection blood to be definitely lower than the control blood. The return to the preinjection level shows considerable animal variation but is usually rapid. The size of the dose seems to be the chief controlling factor in the time taken for recovery. The rabbits receiving 200 mg. per kilo, only two of which are included in Table V, invariably show a marked drop in blood glutathione. As shown in Table IV, the lowest point in the rabbits injected with 200 mg. per kilo is frequently zero in blood taken at the end of the injection. Fig. 4 illustrates the average effect of alloxan on blood reduced glutathione during 24 hours subsequent to the injection of 200 mg. per kilo. The data included here have been compiled from a total of 55

TABLE V

Effect of Gradient Doses of Alloxan on Blood Glutathione in Rabbits

Rabbit No	Alloxan injected	Blood reduced glutathione, mg per 100 cc				Blood sugar, mg per 100 cc				Development of diabetes mellitus
			After injection				After injection			
		Control	10 min	30 min	24–48 hrs	Control	30 min	24–48 hrs	1–2 wks	
	mg per kg									
127	20	58.6	47.7	50.5		116	109	114	95	None
128	40	38.8	35.0	33.8		116	120	112	99	"
101	60	55.8	54.4	53.1	46.1	110		135	120	"
102	60	45.4	42.5	45.4	40.6	130	160	129 101	133	"
103	80	35.5	46.5	46.1	44.7	117	191	130	126 185	"
117	80	33.8	30.7	36.3		128	135	119 142	328 232	Definite diabetes
119	80	45.8	22.9	16.5		111	154	119	112	None
104	100	48.0	46.8	48.0	47.2	111	160	122	488 580	Definite diabetes
109	100	50.6	38.6	55.3	39.9 42.0	117	183	240 164	314 181 132	Transitory diabetes
111	100	39.5	26.5	38.6	42.5	125	178	230 356	392	Definite diabetes
112	100	44.7	40.3	37.1	45.6	115	157	500	Died	" "
105	125	52.0	38.9	32.8	50.4	95	140	512 444	356	" "
113	125	42.7	28.1	32.5	44.0	105	156	362	390 131	Transitory diabetes
106	150	52.0	24.5	44.0	40.0	113	131	270	416 180 129	" "
107	150	57.0	44.6	46.5	60.0	115	238	322 438	Died	Definite diabetes
108	150	45.8	32.7	40.8	52.0	131	227	109	444 516	" "
87	200	43.7	24.5	38.8	40.0*	109	238†	460*	Killed	" "
79	200	39.4	27.8	44.3		85	90	322	580	" "

* 5 hours.

† 2½ hours.

rabbits, and in each time group the average has been determined from the calculated percentages of the control values.

The relationship between the glutathione drop and the tendency of the animal to develop diabetes is not always definite. (As a criterion for the development of diabetes we have arbitrarily chosen throughout all our work two blood sugars taken at different times with values over 200 mg. per 100 cc. with glycosuria. Although with doses of alloxan under 200 mg. per kilo there is a definite tendency for the animals to show a transitory type of diabetes existing for shorter or longer intervals, we believe that during the periods in which the animal shows at least two high blood sugars it is truly diabetic.) In doses under 80 mg. per kilo no diabetes developed and these animals show little change in glutathione. In 80 mg. per kilo doses in three rabbits, one showed the characteristic glutathione 10 minute drop and another one developed high blood sugars 2 weeks after injection. In doses of 100 to 125 mg. per kilo, in six rabbits all showed diabetes, although in this group a definite tendency was observed toward the transitory type of diabetes reported before. This group shows a definite glutathione drop in four of six rabbits in bloods taken at the 10 minute period With doses of 150 and 200 mg per kilo the production of diabetes is almost invariable and the characteristic initial drop in blood reduced glutathione is observed in every case. If one infers that a critical point may exist as regards glutathione changes and the development of diabetes, our data indicate this point to be in the neighborhood of an alloxan dose of 100 mg. per kilo of body weight.

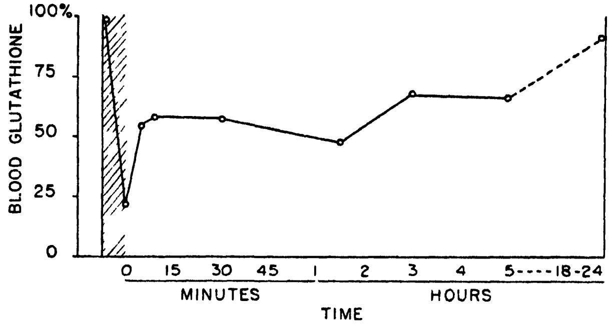

Fig 4 Average reduced glutathione of blood following injection of alloxan The shaded area represents the period of injection Zero minute represents blood taken from the animal as the injection was being completed Scale for first hour, magnified 4 times

In a third series four rabbits were injected with small doses of alloxan and followed for blood glutathione changes during a period of days as diabetes was developing. The first rabbit, No. 58, in this series received 40 mg. per kilo daily. On the 18th and 20th days after the injections started, this rabbit's blood sugar had reached a level of 160 and 165 mg. per 100 cc., with blood reduced glutathione having increased from control values of 38.1 and 37.5 mg. per 100 cc. to 59.5 and 53.9 mg. per 100 cc. respectively. On the 21st day the glutathione was 65.2 mg. per 100 cc. and on the 22nd day the blood sugar reached the diabetic level of 268 mg. per 100 cc., with the glutathione falling to 48.4 mg. per 100 cc. Unfortunately this rabbit was injured from a heart puncture and died as a consequence of taking the last sample of blood.

Rabbit 73 received 40 mg. per kilo intravenously for 16 days and then received seven subcutaneous doses of 60 mg. per kilo. Blood sugar changes and glutathione changes in this rabbit are shown in Fig. 5.

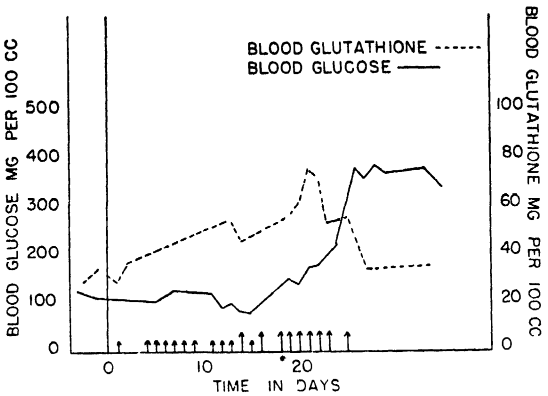

Fig 5 Blood sugar and blood reduced glutathione during development of alloxan diabetes in Rabbit 73 The shorter arrows represent 40 mg of alloxan per kilo; the longer arrows, 60 mg. of alloxan per kilo Subcutaneous injections started at the point indicated by the asterisk

Rabbit 101 received four 60 mg. per kilo injections with 12 days elapsing between the first two injections and 3 or 4 days between the remaining doses. Definite diabetes developed after the fourth dose. In this rabbit, although some fluctuation occurs in glutathione values, there is no general rise as shown in Rabbits 58 and 73.

Rabbit 102 has received five 60 mg. per kilo doses at intervals of 3 to 4 days. There has been a tendency for increased blood glutathione and also a transitory type of diabetes, but at the present time no permanent diabetes has developed.

Simultaneous Injection of Alloxan and Reduced Glutathione—Since two of the rabbits studied as diabetes was developing gave some evidence of glutathione acting as a protective agent, this possibility has been tested in four rabbits by injecting large amounts of glutathione simultaneously with alloxan.

Rabbits 129 and 131 received an intravenous injection of 400 and 300 mg. per kilo respectively of reduced glutathione simultaneously with the injection of 200 mg. per kilo of alloxan. Both rabbits died within 12 hours, and histological sections showed that the glutathione did not protect the islets of Langerhans from the destructive effects of alloxan. The islet changes were comparable to those found at similar periods after the injection of alloxan alone.

Rabbit 124 was given 200 mg. per kilo of alloxan and the same dose intravenously of reduced glutathione. Diabetes developed in the usual manner in this rabbit.

Rabbit 130 was given 300 mg. per kilo of reduced glutathione with the simultaneous injection of 150 mg. per kilo of alloxan. The latter dose of alloxan alone had previously produced permanent diabetes in two rabbits and transitory diabetes in one (Table V). In this rabbit no diabetes developed. Further investigation is in progress along these lines, but our present evidence would indicate that reduced glutathione in doses of either 400, 300, or 200 mg. per kilo does not protect the animal from the diabetogenic action of 200 mg. of alloxan per kilo.

DISCUSSION

The experimental data presented indicate that a large share of injected alloxan is probably almost instantly destroyed by the natural neutralizing factors of the blood and by interaction with glutathione. Whether a small fraction which is not destroyed reaches the pancreas intact and acts directly on the islets of Langerhans has not been proved. Our experiments would indicate that sufficient time elapses before destruction of alloxan is complete to make such direct action possible.

It is of interest to note here that in two experiments an alloxan solution, neutralized with sodium hydroxide to a pH of 7.4 just prior to injection, failed to produce diabetes when injected in the standard diabetogenic dose of 200 mg. per kilo. Also in two experiments *no diabetes was produced* when the standard diabetogenic dose of alloxan was allowed to be in contact with rabbit plasma in one instance and with human plasma in another instance for several hours and then injected into rabbits. These experiments, taken in conjunction with our observations on the rapidity with which our color reaction for alloxan in the blood stream becomes negative, indicate that the diabetogenic activity of alloxan is related to reactions involving the intact alloxan molecule.

It seems logical to believe that the active destructive factors observed in the blood will be equally effective in destroying alloxan in the tissues. Preliminary work on tissue glutathione gives some evidence that this is the case. Although Liebig (13) and Lang (14) reported the presence of alloxan in human urine and in intestinal mucus under pathological conditions, all of our attempts to determine alloxan as such in tissues have been unsuccessful. Unfortunately, neither of the methods described is sufficiently specific to be of value in tissue analysis at the present time.

It is rather difficult from our present data to interpret the results obtained from the experiments attempting to relate blood changes in reduced glutathione with the development of diabetes. In general, we may conclude that with the diabetogenic dose of alloxan the blood glutathione is always rapidly and sharply depressed for a while. With smaller doses of alloxan and slowly developing diabetes, correlations of blood reduced glutathione and onset of the disease are more difficult to make and must await additional experimentation.

TABLE VI

Blood Reduced Glutathione and Blood Thioneine in Two Rabbits after Injection of Alloxan, 200 Mg. per Kilo

Rabbit No	Blood reduced glutathione, mg per 100 cc				Blood thioneine, mg per 100 cc *			
	Control	After injection			Control	After injection		
		0 min	10 min	30 min		0 min	10 min	30 min.
121	49.9	25.8	30.3	30.3	0.59	0.37	0.33	0.49
122	49.4	28.8	21.1		0.43	0.35	0.31	

* Expressed as uric acid thioneine (11).

If the action of alloxan which has been observed with glutathione is a general reaction of alloxan acting as a hydrogen acceptor with sulfhydryl groups, one would expect the action not to be limited to reduced glutathione but to include other sulfhydryl compounds in the animal body. In two rabbits we have shown that the blood glutathione drop is directly paralleled with a drop in thioneine (ergothioneine) (Table VI).

If both glutathione and thioneine react with alloxan in the animal organism it is quite probable that the sulfhydryl group of cysteine may also be involved and work at present is being directed toward determining the effect of alloxan on cysteine and cystine. Increasing evidence that alloxan in small doses may produce a transitory type of diabetes suggests that periods occur in the development of alloxan diabetes consistent with a condition of a reversible equilibrium of some kind in the animal. Histological evidence for the temporary existence of such a reversible state has already been demonstrated in findings previously reported of reversible cellular changes in very early diabetes (15). Perhaps the understanding of the mechanism of alloxan diabetes lies in the understanding of all of the controlling factors of this reversible equilibrium. At least, since cystine forms an integral part of the insulin molecule, the idea of alloxan in large doses causing a sudden and profound disturbance in the cystine-cysteine equilibrium presents an interesting theory.

SUMMARY

1. A quantitative method for the determination of alloxan in blood is presented. This method is based on the ability of alloxan to reduce ferricyanide in the cold.

2. A qualitative color test for alloxan in blood based on its ability to reduce tungstic acid at room temperature is described.

3. Blood alloxan determinations in twelve rabbits injected with a 200 mg. per kilo dose indicate that alloxan, as determined by its reducing ability, disappears almost completely from the blood within 2 minutes. The point of highest concentration is at the end of the injection period.

4. Blood alloxan determinations and blood reduced glutathione determinations in six rabbits injected with 200 mg. per kilo indicate that there is an immediate reaction between alloxan and blood reduced glutathione with the tendency for the complete disappearance of both.

5. A study of blood reduced glutathione changes in relation to the development of alloxan diabetes in rabbits receiving gradient doses of alloxan indicates that the critical level where blood reduced glutathione changes tend to parallel development of diabetes is about 100 mg. per kilo.

6. A study of blood glutathione changes in four rabbits during development of alloxan diabetes through repeated small doses of alloxan suggests that blood glutathione tends to be increased over normal during the development of diabetes.

7. The intravenous injection of reduced glutathione does not appear to protect the rabbit from the diabetogenic effect of simultaneous injection of 200 mg. per kilo of alloxan.

8. In two rabbits 200 mg. of alloxan per kilo caused a decrease in blood thioneine, paralleling the decrease in blood glutathione.

Addendum—Some recent samples of alloxan have yielded glucose equivalents of 128 to 130 instead of the values 115 to 120 given in the text on p. 530 Since writing the paragraph on p 540, we have had success in demonstrating alloxan as such in the tissue of the pancreas in quantities 24 to 56 mg. per cent at the end of the injection

BIBLIOGRAPHY

1. Bailey, C. C., and Bailey, O. T., *J. Am. Med. Assn.*, **122**, 1165 (1943).
2. Brunschwig, A., Allen, J. G., Goldner, M. G., and Gomori, G., *J. Am. Med. Assn.* **122**, 966 (1943).
3. Goldner, M. G., and Gomori, G., *Endocrinology*, **33**, 297 (1943).
4. Dunn, J. S., Kirkpatrick, J., McLetchie, N. G. B., and Telfer, S. V., *J. Path. and Bact.*, **55**, 245 (1943).
5. Dunn, J. S., and McLetchie, N. G. B., *Lancet*, **2**, 384 (1943).
6. Lieben, F., and Edel, E., *Biochem. Z.*, **244**, 404 (1932).
7. Folin, O., *J. Biol. Chem.*, **77**, 421 (1928).
8. Folin, O., and Malmros, H., *J. Biol. Chem.*, **83**, 115 (1929).
9. Labes, R., and Freisburger, H., *Arch. exp. Path. u. Pharmakol.*, **156**, 226 (1930)
10. Benedict, S. R., and Gottschall, G., *J. Biol. Chem.*, **99**, 729 (1932-33).
11. Potter, V., and Franke, K., *J. Nutr.*, **9**, 1 (1935).
12. Schelling, V., *J. Biol. Chem.*, **96**, 17 (1932).
13. Liebig, J., *Ann. Chem.*, **121**, 80 (1862).
14. Lang, G., *Wien. med. Wochschr.*, **16**, 1513 (1866).
15. Bailey, C. C., Bailey, O. T., and Leech, R S., *New England J. Med.*, **230**, 533 (1944).

VITAMIN INTERRELATIONSHIPS

IV. FURTHER STUDIES ON THE INFLUENCE OF CHRONIC THIAMINE DEFICIENCY ON RIBOFLAVIN METABOLISM*

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In 1942 Sure and Ford (1) demonstrated that in acute thiamine deficiency there is a marked reduction in the utilization of riboflavin because of poor retention. Ferrebee and Weissman (2), however, contend that the large excretions of urinary riboflavin we encountered in thiamine avitaminosis were produced by body tissue catabolism. That this is not the case became evident recently (3) when large urinary riboflavin excretions were observed in chronic thiamine deficiency in animals kept at maintenance on suboptimum doses of thiamine compared with animals fed isocaloric diets, but which received a sufficiency of vitamin B_1. In order to obtain further information on thiamine-riboflavin interrelationships, a study was begun in March of this year on the influence of chronic thiamine deficiency on riboflavin tissue metabolism with the further objective of securing fecal as well as urinary riboflavin balances, in order to obtain complete information on riboflavin utilization. In the meantime the work of Singher and associates (4) appeared, in which they presented evidence that during a depletion period of 24 to 28 days the concentration of riboflavin in the liver of thiamine-deficient rats was higher than in control animals. From such findings they concluded that in vitamin B_1 deficiency there are greater riboflavin reserves available and they, therefore, disagreed with the postulation of Sure and Ford (1) that there is poorer utilization of riboflavin in thiamine deficiency. Contrary to the findings of Singher and coworkers, Ferrebee and Weissman (2) found a lower concentration of riboflavin in liver and kidney in thiamine-deficient rats than in controls; neither was an increase in riboflavin content of the liver observed in thiamine deficiency by Supplee and associates (5).

In view of the conflicting literature, it is particularly timely to submit our detailed data on riboflavin utilization and its concentration in all tissues, including the endocrines, in thiamine deficiency. Since border line rather than marked thiamine deficiencies are more commonly found in this country, this investigation was limited to observations in the chronic state.

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EXPERIMENTAL

The experiments were carried out on three groups of albino rats, twenty-four in each group. In each group one-half served as thiamine-deficient and one-half as control. Before the termination of the experiments one vitamin B_1-deficient rat died in Group A; therefore, its control had to be eliminated, leaving twenty-two animals in this group. Group A consisted of males which were started on experiments when 43 days old and weighed 60 to 76 gm. each. Group B consisted of females which were 56 days of age and weighed 68 to 88 gm. each when placed on experiments. Group C consisted of an equal number of males and females. They were started on experiments when 51 days old and weighed 72 to 82 gm. each. The animals received a purified synthetic diet of the following composition: casein (vitamin-free Smaco) 18, Cellu flour 2, Salts 1 (6) 4, butter fat 5, and cerelose 71. As a source of vitamins A and D 3 drops of halibut liver oil, and as a source of vitamin E 1 drop of a tocopherol concentrate, were given once weekly to each animal. In each group the avitaminotic animal was allowed 2 to 4 γ of thiamine daily, which was sufficient to keep it from developing the characteristic symptoms of polyneuritis The control animals were given daily 20 γ of thiamine and isocaloric diets consumed by the vitamin B_1-deficient rats. Other vitamin supplements were given daily, separately from the ration, as follows: 20 γ of pyridoxine, 6 mg. of choline chloride, and 200 γ of calcium pantothenate. The animals in Group A received 10 γ of riboflavin daily for 187 days. When it became apparent that it was impossible to prevent losses of weight in the B_1-deficient rats on the daily dose of thiamine which generally was sufficient for the prevention of polyneuritis, the daily dose of riboflavin was increased to 20 γ. Maintenance in the thiamine-deficient animals was immediately established when a 14 day riboflavin balance study was conducted. The animals in Group B received 50 γ of riboflavin daily for 202 days, on which a 2 week riboflavin balance was carried out at the termination of the feeding period. Group C was given only 5 γ of riboflavin daily for 172 days, in order to provide information on riboflavin excretions on a diet deficient in this vitamin. It was possible then to reduce further the daily riboflavin intake to 3 γ without subsequent losses of body weight for a period of 14 days when data were obtained on fecal and urinary excretions of riboflavin. These rats were then given 100 γ of riboflavin daily for another 14 days, during which period riboflavin balances were secured. Following the completion of the riboflavin balance experiments the animals were sacrificed, and the different tissues and endocrines from all the deficient and all the control groups were pooled and analyzed according to methods previously described (1). The urinary determinations were made according to modified technique recently described (3). In order further to reduce the possibilities of bacterial synthesis in the feces (1, 7), the latter were collected twice daily, at 8 a.m. and 4.30 p.m., then covered with petroleum ether in amber-colored bottles, and kept in an electric refrigerator at about 1° for a week until they were ready for analysis. Our results are summarized in Tables I, II, and III.

DISCUSSION

It is apparent from Table I that chronic thiamine deficiency produces no disturbance in absorption of riboflavin, as evidenced from fecal excretions.

TABLE I

Influence of Chronic Thiamine Deficiency on Riboflavin Utilization

The figures represent averages per animal. The period of metabolism was 14 days in each group.

Group	Animals	Change in weight during metabolism period	Change in weight during entire experimental period	Daily riboflavin intake	Total riboflavin intake	Riboflavin excreted in feces*	Riboflavin absorbed		Riboflavin excreted in urine*	Riboflavin retained	Riboflavin utilized
		gm	*gm*	γ	γ	γ	γ	*per cent*	γ	γ	*per cent*
A	11 Deficient	+2.6	+58.4	20	280	48.6	231.4	82.3	69.3	162.1	70.5
	11 Control	+1.3	+97.7	20	280	58.6	221.4	79.1	23.9	197.5	88.2
B	12 Deficient	+0.8	+40.6	50	700	38.0	662.0	94.5	145.2	516.8	78.1
	12 Control	+2.8	+78.6	50	700	46.6	653.4	93.3	56.2	597.2	91.9
C	12 Deficient	+1.8	+37.5	100	1400	50.6	1349.4	96.4	267.2	1082.2	80.2
	12 Control	+1.6	+76.4	100	1400	105.4	1294.6	92.5	161.2	1133.4	87.6

*Corrected for the amount excreted on a riboflavin-deficient diet.

However, as recently demonstrated (3), in the same state of B_1 avitaminosis, uncomplicated by body tissue catabolism, there are large increases in the urinary excretion of riboflavin compared with those found in control rats. On the 20 γ, 50 γ, and 100 γ riboflavin daily doses, there were reductions in utilizations of riboflavin of 17.7 per cent, 13.8 per cent, and 7.4 per cent respectively. It is evident then that the animal organism requires higher intakes of riboflavin for its optimum utilization when the intake of thiamine is inadequate.

The greater gains in body weight by the control animals, which received 20 γ of thiamine daily on the isocaloric diets consumed by the avitaminotic rats given 2 to 4 γ of vitamin B_1 daily, are in accord with our previous findings (8–10) and with those of Whipple and Church (11), with the results of Graham and Griffith (12), and with those of Mitchell (13), that thiamine exerts a specific effect on growth. Therefore, when the animals were sacrificed for analysis of tissue riboflavin, the vitamin B₁-deficient rats were of

Table II

Influence of Chronic Thiamine Deficiency on Riboflavin Content of Various Tissues of the Albino Rat

Tissue	Group A, 20 γ riboflavin daily				Group B, 50 γ riboflavin daily				Group C, 100 γ riboflavin daily			
	Deficient		Control		Deficient		Control		Deficient		Control	
		Total in entire tissue*		Total in entire tissue*		Total in entire tissue†		Total in entire tissue†		Total in entire tissue‡		Total in entire tissue‡
	γ per gm	γ	*γ per gm*	γ	*γ per gm*	γ	*γ per gm*	γ	*γ per gm.*	γ	*γ per gm*	γ
Muscle	1.8	80.7	1.5	88.8	3.7	180.2	4.0	292.0	3.3	161.7	4.0	280.0
Liver	45.6	643.0	37.5	545.6	60.0	724.2	76.7	1077.6	80.0	999.2	56.7	846.0
Kidney	55.5	211.5	56.6	190.7	76.7	280.0	83.3	271.6	76.7	246.2	66.7	210.1
Spleen	28.8	18 4	8.8	8.5	5.6	5.0	10.6	12.5	12.5	11.1	8.8	10.6
Heart	43.1	64.2	19.4	27.9	46.9	48.8	53.3	82.2	34.2	44.5	22.2	35.5
Lung	16.9	31.3	8.3	20.7	13.2	20.5	11 1	30.4	15 5	29.5	8.3	22.4
Stomach	23.6	43.7	8.3	18.3	13.3	29.4	13 3	30.3	10 0	20.6	9.1	22.0
Pancreas	23.6	43.2	12.3	26.8	15.8	32.1	24.2	50 3	22 5	46.6	20.8	44.2
Small intestines	6.1	24.8	9.4	37.6	12.6	52.5	16.1	72.3	14.4	63.8	8.3	41.0
Large intestines	12.8	34.6	4 4	10.7	11.1	32 2	11.7	29 3	12.2	34.0	7 8	21.1
Brain	8.3	21.9	8.3	22.3	7.8	22.2	12.7	38.1	6.7	18.9	7.7	21.4
Testes	13.9	30.2	7.2	16.4	7.5	10.1	11.7	18.7				
Ovaries					17.7	0 7	33.7	2.3	27.4	4 3	22.2	4.0
Thymus	25.0	3.8	8.5	2.0	36.2	4.1	33.3	10.0	18.0	2.8	23.4	4.3
Adrenals	22.4	1.7	31.0	1.8	30.0	2.4	28.7	2.7	23 4	1.8	27.8	2.7
Thyroids	10.0	0.3	8.7	0.3	25.0	0.7	26.9	0.9	21.2	0.7	16.7	0.6
Pituitary	20.8	0.2	30.3	0.2	31.3	0.2	33.3	0.3	30.9	0.4	25.0	0.3
Total in all tissues		1253.5		1018.6		1445.3		2021.5		1686.1		1566.2

* The tissues from this group were taken from eleven deficient and eleven control rats.

† The tissues from this group were taken from twelve deficient and twelve control rats. However, since half of these animals were males and half females, the ovaries and testes represent six of the deficient and six of the control rats.

‡ The tissues from this group were taken from twelve of the deficient and twelve control rats.

smaller size, weighing an average of 118 gm. each compared with 160 gm. each for the control animals.

In Table II data are presented on the concentration of riboflavin per gm.

of all the tissues and endocrines, as well as the total riboflavin content in the entire animal body of the B_1-deficient and control rats. While the concentration of riboflavin in the liver is higher in the thiamine-deficient animals in Group A on the 20 γ daily dose and in Group C on the 100 γ daily intake, the reverse picture is found in Group B on the 50 γ daily allowance of riboflavin. The significant point about the vitamin content of a tissue is not the concentration per gm. but the total amount in the entire tissue which, as in the case of the muscles, is dependent on the size of the animal which determines the weight of the muscles. In the evaluation of all the riboflavin tissue data, it should be borne in mind that the control animals at the termination of the experiments weighed about 26 per cent more than the thiamine-deficient rats on isocaloric diets and the same riboflavin intake. When we consider the total amount of riboflavin in the liver, kidneys, heart, and muscles (which represent over 80 per cent of the total tissues) of the avitaminotic and control animals of all the groups, an entirely different picture is apparent. The following are the average figures:[1] *liver*, deficient, 2366; control, 2469; *kidney*, deficient, 736; control, 672; *heart*, deficient, 158, control, 146; *muscles*, deficient, 423; control, 661. It is evident then that there are no noteworthy differences in the riboflavin stored in the liver,

TABLE III

Influence of Chronic Thiamine Deficiency on Riboflavin Metabolism

The figures represent averages per animal. The metabolism period was 14 days in each group.

Group	Daily riboflavin intake	Total riboflavin intake during metabolism period	Riboflavin excreted in feces	Riboflavin excreted in urine	Total riboflavin intake during entire experimental period	Riboflavin in tissues	Riboflavin stored in tissues during entire experimental period
	γ	γ	*per cent of total intake*	*per cent of total intake*	*per cent of total intake*	γ	*per cent of total intake*
A, deficient	20	280	17.4	24.8	2,150*	114	5.3
" control	20	280	20.9	8 5	2,150*	93	4.3
B, deficient	50	700	5.5	20.7	10,100†	120	1.2
" control	50	700	6.6	8.0	10,100†	168	1.6
C, deficient	100	1400	3.6	19.0	2,332‡	141	6.1
" control	100	1400	6.1	11.5	2,332‡	131	5.7

* This group received the following doses of riboflavin per animal per day: 10 γ for 187 days and 20 γ for 14 days.

† This group received 50 γ of riboflavin per animal per day for 202 days.

‡ This group received the following doses of riboflavin per animal per day 5 γ for 172 days; 3 γ for 14 days, 100 γ for 14 days.

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[1] Expressed in micrograms.

kidney, and heart between the thiamine-deficient and the control rats. There is, however, a marked reduction in the riboflavin of the muscles of the vitamin B_1-deficient animals. An examination of the weights of the different tissues disclosed that there were significant differences only in the weights of muscle tissue, which was 25 per cent less in the vitamin B_1-deficient than in the control rats. Since there was a reduction of 36 per cent in muscle riboflavin in the avitaminotic animals and the latter had 25 per cent less muscle than the control rats, about 70 per cent of reduction of riboflavin should be attributed to differences in weight of tissue. Our data do not warrant the conclusion that the reduced reduction of riboflavin in the muscles is responsible for the increased urinary excretions of this vitamin in the thiamine-deficient animals.

It is apparent from an analysis of the data submitted in Table II that the higher concentration of riboflavin per gm. of liver in thiamine-deficient rats than in control animals reported by Singher and associates (4) is of no significance in relation to riboflavin metabolism of the animal organism. While liver is a specific storage organ for vitamins A and D, which the animal can draw on for reserves during periods of inadequate intake of these vitamins, it is not a specific depot for riboflavin and other components of the vitamin B complex. According to Supplee and coworkers (5), "The riboflavin concentration in the liver increases during digestion and assimilation, being mobilized therein presumably from other tissues; this transient concentration takes place even in animals whose tissue stores have been impoverished by a prolonged riboflavin-free dietary."

Recently Sure and Ford (7) demonstrated that when rats are given 1000 γ of riboflavin for 30 days, which provided 30,000 γ of this vitamin, only about 800 γ were found in the animal body, which constitutes a storage of only 2.6 per cent. From Table III it is evident that for the entire experimental period the per cent of riboflavin stored in the body is only 2:6, the greater part of the total intake of which, it was recently shown, is destroyed by the tissues (7). It is also apparent from Table III that the urinary excretions of riboflavin in chronic thiamine deficiency are 2 to 3 times as great as in the control animals.

Table II shows that, while in Group A the total riboflavin content in the tissues of the thiamine-deficient animals is 25 per cent higher than in the controls, in Group B the riboflavin content of the tissues is 40 per cent higher in the control than in the avitaminotic rats, and in Group C there was no noteworthy difference between the vitamin B_1-deficient and control animals. Therefore, it must be concluded that, on the whole, the riboflavin content of the tissues, which represents only a small proportion of the total intake of this vitamin during the entire experimental period, has not been influenced by chronic thiamine deficiency for a period of over 6 months.

Also, since suboptimum thiamine intakes have had no influence on riboflavin absorption but produced a marked effect on urinary excretions of riboflavin, the latter results account for poorer utilization of this vitamin in chronic vitamin B_1 deficiency. The poorer utilization of riboflavin in chronic thiamine deficiency is, however, not as great as in acute thiamine deficiency (1).

SUMMARY

Chronic thiamine deficiency produces no noteworthy differences in riboflavin content of body tissues nor in riboflavin absorption compared with those found in control rats. There is poorer utilization of riboflavin in chronic thiamine deficiency than in control rats on isocaloric diets, receiving the same intake of riboflavin, but the poorer utilization is not as great as in acute vitamin B_1 deficiency.

BIBLIOGRAPHY

1 Sure, B, and Ford, Z W, Jr, *J. Biol Chem*, **146,** 241 (1942)
2 Ferrebee, J W, and Weissman, N, *J Nutr*, **26,** 459 (1943)
3 Sure, B, *J Nutr*, **27,** 447 (1944)
4 Singher, H O, Kensler, C J, Levy, H, Poore, E, Rhoads, C P, and Unna, K, *J Biol Chem*, **154,** 69 (1944)
5 Supplee, G C, Jensen, O G, Bender, R C, and Kahlenberg, O J, *J Biol Chem*, **144,** 79 (1942)
6 Sure, B, *J Nutr*, **22,** 499 (1941)
7 Sure, B, and Ford, Z W, *J Nutr*, **26,** 659 (1943)
8 Sure, B, Kik, M C, Smith, M E, and Walker, D J, *Science*, **73,** 285 (1931)
9 Sure, B, Kik, M C, and Smith, M E, *J Nutr*, **5,** 155 (1932)
10 Sure, B, *J Biol Chem*, **97,** 133 (1932)
11 Whipple, D V, and Church, C F, *Proc Am Soc Biol Chem*, *J Biol Chem*, **119,** p ciii (1937)
12 Graham, C E, and Griffith, W H, *J Nutr*, **6,** 195 (1933)
13 Mitchell, H H, *Am J Physiol*, **104,** 594 (1933)

RELATIVE EFFECTS OF CASEIN AND TRYPTOPHANE ON THE HEALTH AND XANTHURENIC ACID EXCRETION OF PYRIDOXINE-DEFICIENT MICE*

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Musajo (1) first isolated xanthurenic acid from the urine of rats fed diets high in fibrin, and later characterized the compound as 4,8-dihydroxyquinoline-2-carboxylic acid (2). Its solutions gave an intense green color with iron salts, and the compound was found in the urine of rats, guinea pigs, or rabbits after the administration of tryptophane or kynurenine, but not after kynurenic acid (3, 4) Lepkovsky and Nielsen (5) noted that the urine from pyridoxine-deficient rats also formed a green color with ferric iron. The chromogen was excreted by deficient dogs (6) and pigs (7), but not by deficient chickens (8) Lepkovsky, Roboz, and Haagen-Smit (8) isolated the chromogen from the urine of deficient rats and presented evidence that it was xanthurenic acid The excretion of the chromogen increased when *l*-tryptophane or kynurenine was fed (8, 9), and when pyridoxine was administered, the excretion of xanthurenic acid ceased The chromogen failed to appear in the urine of pyridoxine-deficient rats fed acid-hydrolyzed casein or zein and gelatin. From these results Lepkovsky *et al* (8) concluded that "the function of pyridoxine is related to tryptophane metabolism"

The question remains whether pyridoxine is concerned solely with tryptophane or whether the metabolism of other amino acids is also abnormal in pyridoxine deficiency. Cerecedo and Foy (10) added tryptophane to a pyridoxine-deficient diet containing 15 per cent of casein without decreasing the time necessary for the appearance of the characteristic dermatitis in rats. Nevertheless the syndrome developed more rapidly on high casein diets (11, 12), and the addition of cystine increased the severity of the dermatitis (10). Fishman and Artom (13) found that pyridoxine minimized the injurious effect of large amounts of serine given parenterally or by stomach tube. Pyridoxine (14) and pyridoxal (15) also increased the rate of tyrosine decarboxylation by a cell suspension of *Streptococcus faecalis* R.

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The present report deals with the quantitative determination of xanthurenic acid in urine and with the relative effects of protein and tryptophane on the health and chromogen excretion of mice fed diets low in pyridoxine.

Methods

Care of Mice—Weanling albino or C_3H mice were kept in groups of four in screen bottom cages. Food and water were given *ad libitum*, and the animals were weighed at weekly intervals. The diets (Table I) contained 10 to 60 per cent of purified casein,[1] 4 per cent of salts (17), 2 per cent of corn oil (Mazola), and glucose[2] to 100 per cent. One part of halibut liver oil was added to 1000 parts of the corn oil. A synthetic vitamin mixture (Table I) was added to all of the diets at a constant level, except that the pyridoxine hydrochloride[3] varied from 0 to 10 γ per gm. of diet. Prior to the experiments proper the mice were maintained on suitable synthetic diets of known vitamin content. In experiments on the relative effects of various levels of casein and pyridoxine, the preliminary ration, which was fed for 6 days, contained 20 per cent of casein and 10 γ of pyridoxine per gm. of diet. In experiments on the effect of individual amino acids

TABLE I

Composition of Diets

	per cent
Purified casein	10, 20, 30, 45, or 60
Corn oil + 0.1% halibut liver oil ..	2
Wesson's salt mixture	4
Glucose	To 100
	γ per gm
Pyridoxine hydrochloride	0, 0 5, 1, 2, 5, or 10
Thiamine chloride	3 3
Nicotinic acid	10 0
Calcium pantothenate	13 3
Riboflavin	6 6
Choline chloride	166 0
Inositol	333 0
p-Aminobenzoic acid	200 0

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[1] Crude casein was washed for 1 week with several changes of tap water and then extracted with two changes of ethyl alcohol for 4 days at 50°. The purified casein contained 0.2 γ of pyridoxine hydrochloride per gm according to assays with *Saccharomyces carlsbergensis* (16).

[2] Cerelose, a pure commercial glucose monohydrate. It contained less than 1 millimicrogram of pyridoxine hydrochloride per gm. (16).

[3] In the present report the terms pyridoxine and pyridoxine hydrochloride are used interchangeably, and all values are expressed as the hydrochloride.

the mice were depleted of pyridoxine for about 2 weeks on a diet containing 10 per cent of casein and no pyridoxine before the various experimental diets were fed.

For the collection of urine each group of mice was placed periodically in a metabolism cage fitted with an outside feeder. The funnels were of glass, and the floors of the cages as well as the fine mesh screening in the funnels were of galvanized iron. The urine was collected under a small amount of benzene After 24 hours the funnels were rinsed with 5 to 10 cc. of distilled water, and the washings added to the urine, which was then stored in the refrigerator until analysis. Since the precursors of the chromogen appeared to be of dietary origin, the quantity of food consumed by each group was determined during the 24 hours prior to each urine collection. The level of excretion was then expressed as micrograms of xanthurenic acid per gm. of diet consumed.

Determination of Xanthurenic Acid[4] *in Urine*—The basis of this determination was the green color formed by a solution of xanthurenic acid in the presence of ferric chloride. A qualitative test (1, 8) could easily be carried out by the addition of ferric chloride to alkaline urine, but this procedure failed to give quantitative results. For maximum color development it was necessary to add an excess of ferric chloride, and other things being equal, the color intensity increased progressively with the concentration of sodium bicarbonate up to 0 5 per cent. However, a flocculent precipitate formed under these conditions and apparently adsorbed a part of the pigment, since the color intensity of the filtered solution decreased with additional ferric chloride. Furthermore, the color per unit of xanthurenic acid increased with increasing concentrations of the chromogen.

Hence the following procedure was adopted. The urine was acidified to pH 2 5 ± 0 5 (external indicator), and the total volume recorded to ±0 1 cc. The urine was filtered immediately through a qualitative paper, and a 2 cc. aliquot of the filtrate pipetted into a 50 cc. test-tube containing 0 4 gm. of sodium chloride. 25 cc. of a 15:1 mixture of ethyl ether and ethyl alcohol were then added and the mixture was stirred vigorously for 5 minutes with a mechanical stirrer. For routine determinations a multiple stirring unit was used. The ether-alcohol layer was separated from the aqueous layer and transferred to a flask. The solvent was then dried with 4 gm. of anhydrous sodium sulfate and transferred to a clean flask. The sodium sulfate was washed three times with 5 cc. of ether-alcohol, the washings were added to the urine extract, and the solvent was removed *in vacuo* (water bath 60–70°). The residue was then dissolved in 9 cc. of absolute ethyl alcohol,[5] and 1 cc. of a 5 per cent solution of ferric chloride in absolute ethyl alcohol was added.

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[4] From the work of Lepkovsky *et al.* (8) it appears probable that in pyridoxine deficiency the principal urinary constituent forming a green color with ferric iron is xanthurenic acid. However, many other compounds also form green complexes with ferric chloride and the possibility of their presence in the urine of either normal or deficient animals cannot be excluded.

The color intensity of this solution was measured with an Evelyn photoelectric colorimeter through the 660 mμ filter after the galvanometer had been adjusted to read 100 with a blank tube containing 9 cc. of absolute ethyl alcohol and 1 cc. of the ferric chloride solution. Under these conditions a single extraction sufficed for most samples of urine. However, when the concentration of the chromogen exceeded 50 γ of xanthurenic acid per cc. of acidified urine, the extraction was repeated. Each extract was then treated separately for color development so that the completeness of extraction could be ascertained.

As a colorimetric standard xanthurenic acid was synthesized according to the method of Musajo and Minchilli (2). This preparation melted at 281–283° (uncorrected). Aliquots of the acid in 9 cc. of alcohol and 1 cc. of the ferric chloride solution yielded a constant, K, of 858 in the relation $C = KL$, where C is the micrograms of xanthurenic acid per tube and L equals $2 - \log G$. However, in the presence of the material extracted from normal urine, the K value increased with the concentration of the urinary material. When a 24 hour sample of urine from four mice was diluted to 15 to 25 cc. and analyzed under the conditions described above, the K values of added xanthurenic acid ranged from 885 to 920. Accordingly, in the present studies, an average constant, $K = 900$, was used for the calculation of the chromogen content of urine. With the latter constant 90 to 99 per cent of either the synthetic xanthurenic acid or of a chromogen concentrate from urine could be recovered routinely in two extractions.

Results

Variations Due to Level of Casein—When 2 γ or more of pyridoxine per gm. of diet were fed to weanling mice, good growth resulted and the level of casein could be varied from 10 to 60 per cent of the diet without affecting either the rate of growth or the survival of the animals[6] (Tables II to IV). However, in the absence of pyridoxine, the condition of the mice depended markedly upon the casein content of the diet. The mice on the higher levels of casein ate less food, excreted more chromogen, lost weight more quickly, and died sooner than those consuming less casein. In a typical series (Table II) mice receiving 60 per cent of casein died in 26 days (16 to 37 days), and those receiving 30 per cent of casein died in 51 days (46 to 56 days). The only death in the group receiving 10 per cent of casein occurred on the 66th day, when the experiment was terminated.

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[5] It is possible to use 95 per cent ethyl alcohol in place of absolute alcohol if the readings are taken immediately after the addition of the ferric chloride to each tube.

[6] When young mice were fed 60 per cent of casein with adequate pyridoxine, growth occasionally ceased for about 1 week (Table III), after which it was resumed. Since this difficulty was not encountered when 45 per cent of casein was fed, the latter level was adopted for the later experiments.

TABLE II

Chromogen Excretion and Growth of Mice Receiving Various Levels of Casein and Pyridoxine (Four Mice Per Group)

Casein	10%		30%		60%	
Pyridoxine per gm of diet	0 γ	10 γ	0 γ	10 γ	0 γ	10 γ
Chromogen excretion, calculated as micrograms of xanthurenic acid excreted per gm. diet consumed						
Basal period*	26*	25*	26*	16*	26*	27*
6th day	54	31	76	51	234	40
11th "	33	13	236	46	598	51
19th "	48	16	461	50	449	33
26th "	61	18	629	29	890	38
60th "	213	15				
Growth, average weight in gm.						
1st day	10.8	11.4	11.3	11.8	11.5	11.2
8th "	10.6	12.7	12.1	13.1	11.4	12.4
15th "	12.3	14.1	12.2	16.4	10.2	14.6
34th "	11.7	17.4	9.8	18.7	7.8	18.4
66th "	10.7	20.7		20.0		21.1

* Obtained on the last day of the 6 day basal period during which the mice received 20 per cent of casein with 10 γ of pyridoxine per gm. of diet.

When the deficient diet contained 60 per cent of casein, the mice excreted 234 γ of xanthurenic acid per gm. of diet on the 6th day and a maximum of 890 γ per gm. on the 26th day (Table II). The excretion of those receiving 30 per cent of casein rose more slowly to a maximum of 629 γ per gm. of diet on the 26th day, while those receiving 10 per cent of casein excreted only 61 γ. All of the groups receiving more than 2 γ of pyridoxine excreted less than 60 γ of xanthurenic acid per gm. of food consumed.

In general the control mice receiving 10 to 30 per cent of casein consumed 2.5 to 3 gm. of diet per day while those on 40 to 60 per cent of casein ate 2 to 2.5 gm. per day. The deficient animals consumed comparable quantities at the beginning of the experiment and decreasing amounts as the deficiency became more severe. After the deficient mice began to lose weight, they drank only 60 per cent as much water as control mice receiving pyridoxine and restricted to the same food intake. About half of the deficient mice on 10 to 30 per cent of casein developed a paralysis of one or both hind legs several days before death. Perhaps because of their more rapid death, this condition was observed less frequently in mice receiving the higher levels of casein. No characteristic dermatitis was observed in any of the mice.

Experiments with Other Proteins—Lepkovsky, Roboz, and Haagen-Smit (8) substituted zein and gelatin or acid-hydrolyzed casein for the casein of their deficient diet and observed that the chromogen disappeared from the urine of deficient rats in 6 to 12 hours. Our attempts to perform similar experiments with mice were unsuccessful. Zein[7] was found to contain 2.7 γ of pyridoxine per gm. (*Saccharomyces carlsbergensis* assay (16)), and the vitamin was not removed by extraction with ether or acetone or by reprecipitation of the zein from alcohol solution with water. Mice fed 40 per cent of zein and 5 per cent of casein therefore received 1.1 γ of pyridoxine per gm. of diet. When this amount of pyridoxine was added to a diet containing 45 per cent of casein, good growth resulted and the excretion of chromogen was low. Thus the failure of chromogen to appear on a zein diet becomes of doubtful significance

Both gelatin and acid-hydrolyzed casein were low in pyridoxine, but these substances appeared to be unpalatable to the mice, the intake of food was low, and weight losses and death occurred even when 10 γ of pyridoxine were added per gm. of diet. The excretion of the chromogen, however, decreased when acid-hydrolyzed casein was substituted for part of the casein of a deficient diet: from 334 γ of xanthurenic acid per gm. of diet to 146 γ.

Variations in Requirement for Pyridoxine—Weanling mice were fed synthetic diets containing 60 or 20 per cent of casein, and the amount of pyridoxine per gm. of diet ranged from 0 to 10 γ (Tables III and IV). The growth of the animals and the excretion of chromogen were then determined periodically. As judged by these criteria 3 to 4 times as much of the vitamin were needed when the diet contained 60 per cent of casein as when it contained 20 per cent. On the former diet the excretion of the chromogen was large and growth was suboptimal when a gm. of diet contained 1.0 γ of pyridoxine (Table III); 2.0 γ or more per gm. of diet appeared necessary to bring growth and excretion within normal limits. When the diet contained 20 per cent of casein, however, growth and

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[7] The zein was obtained through the courtesy of Dr. R. C. Gralow of the Corn Products Refining Company

TABLE III

Chromogen Excretion and Growth of Mice Receiving 60 Per Cent of Casein and 0 to 10 Micrograms of Pyridoxine Per Gm. of Diet (Four Mice Per Group)

Pyridoxine per gm. of diet	0 γ	1 γ	2 γ	5 γ	10 γ
Chromogen excretion, calculated as micrograms of xanthurenic acid excreted per gm. diet consumed					
Basal period*	26*	21*	32*	25*	42*
4th day	246	85	81	64	41
7th "	484	229	86	71	50
14th "	828	435	70	52	24
46th "		395	40	45	46
Growth, average weight in gm.					
1st day	10.9	10.3	11.4	11.1	11.5
8th "	10.8	10.9	12.0	11.3	12.8
18th "	9.9	12.0	14.1	14.8	14.3
54th "		14.3	20.8	19.4	19.8

* Obtained on the last day of the 6 day basal period during which the mice received 20 per cent of casein with 10 γ of pyridoxine per gm. of diet.

TABLE IV

Chromogen Excretion and Growth of Mice Receiving 20 Per Cent of Casein and 0 to 10 Micrograms of Pyridoxine per Gm. of Diet (Four Mice Per Group)

Pyridoxine per gm. of diet	0 γ	0.5 γ	1 γ	2 γ	10 γ
Chromogen excretion, calculated as micrograms of xanthurenic acid excreted per gm. diet consumed					
Basal period*	36*	23*	23*	22*	36*
8th day	84	51	34	24	38
21st "	316	66	39	17	11
51st "	547	93	32	18	34
88th "		61	51	25	18
Growth, average weight in gm.					
1st day	11.2	11.3	10.9	11.8	11.3
19th "	15.0	17.7	17.2	17.6	17.2
57th "	11.8	21.7	23.1	22.0	22.5
89th "		24.3	23.8	23.4	25.1

* Obtained on the last day of the 5 day basal period during which the mice received 20 per cent of casein with 10 γ of pyridoxine per gm. of diet.

excretion were essentially the same on 0.5 γ of pyridoxine per gm. of diet as when higher amounts of the vitamin were fed (Table IV).

Tryptophane—Musajo and Chiancone (3) and Lepkovsky and coworkers (8, 9) showed that tryptophane was the precursor of xanthurenic acid. No attempt was made, however, to determine how much of the dietary tryptophane was excreted in this form by the deficient animals, or to compare the survival of animals on diets high in casein or in tryptophane.

For these purposes weanling mice were fed a diet containing 10 per cent of casein and no pyridoxine for a basal period of 12 days. The mice were then divided into groups of four, a urine collection was made on the basal diet, and the mice were then fed the following diets: 10 per cent of casein, 45 per cent of casein, or 10 per cent of casein plus 0.54 per cent of *l*-tryptophane (Merck). The latter diets were supposed to supply equivalent amounts of tryptophane per gm. of diet. The quantity of *l*-tryptophane added was calculated on the assumption that casein contains 1.6 per cent of this amino acid; the results of chemical analyses range from 1.2 to 2.2 per cent (18–20).

In the absence of pyridoxine the mice receiving 10 per cent of casein plus the *l*-tryptophane consumed more food, appeared in better health, and survived longer than those receiving 45 per cent of casein. The mice receiving only 10 per cent of casein remained in better health and survived longer than either of these groups. As usual the mice on the low casein diet excreted only small amounts of the chromogen while those fed 45 per cent of casein excreted large amounts (Table V). Those receiving the *l*-tryptophane excreted still larger amounts. In a second series the consumption of food by the mice receiving *l*-tryptophane was restricted to that of mice receiving 45 per cent of casein. Nevertheless, the excretion of chromogen per gm. of food remained greater after the ingestion of the pure amino acid than after 45 per cent of casein. The presence of pyridoxine in the diet resulted in normal growth and normal chromogen excretion on all three diets.

Several conclusions can be drawn from this experiment. *l*-Tryptophane decreases the survival of the pyridoxine-deficient mouse, but not so much as casein of equivalent tryptophane content. Mice on the deficient diet containing tryptophane consumed about twice as much food as those on the 45 per cent casein diet and apparently also received somewhat more tryptophane per gm. of diet (see below). Their total consumption of tryptophane was therefore at least twice that of the mice fed the high level of casein. Nevertheless, they survived 50 to 70 per cent longer. The inference is that other amino acids in casein may also be injurious to the pyridoxine-deficient mouse.

The data further suggest that the chromogen might be derived entirely from the tryptophane present in the casein. In the above experiment the tryptophane content of casein was assumed to be 1.6 per cent, but more recently Greene and Black (21) have reported that casein contains only 1.06 to 1.11 per cent of tryptophane as determined with *Lactobacillus arabinosus* 17–5. Analyses by Schweigert *et al.*[8] with the same organism indicate a figure of 1.1 per cent. If these values are more nearly correct than those obtained by chemical methods, it can be calculated that the maximum excretion of xanthurenic acid accounted for 12.2 and 9.6 per cent respectively of the tryptophane ingested as the free acid or as casein. In other series (Tables II to IV) the maximum percentage was 24 per cent when the diet contained 20 per cent of casein, and 13 per cent when it contained 60 per cent of casein.

TABLE V

Chromogen Excretion and Growth of Mice Receiving l-Tryptophane

Casein	10%			45%	
l-Tryptophane added	0	0.54%		0	
Pyridoxine per gm diet.	0 γ	0 γ	10 γ	0 γ	10 γ
Chromogen excretion, calculated as micrograms of xanthurenic acid excreted per gm. diet consumed					
Basal period*	71*	81*	66*	55*	97*
6th day	56	491 (859)†	54	367 (413)	43
12th "	48	793 (1784)	71	460 (575)	30
16th "	23	785 (1470)	69	321 (562)	64
Growth, average weight in gm.					
1st day	11.2	11.4	11.7	11.5	11.8
8th "	11.2	10.8	13.8	10.6	12.6
29th "	10.0	9.8	15.9	9.5	14.6
Survival at 20 days‡	4/4	3/4	4/4	1/4	4/4
" " 36 "	3/4	0/4	4/4	1/4	4/4

* Obtained on the last day of the 12 day basal period during which the mice received 10 per cent of casein and no pyridoxine.

† The figures in parentheses are the total excretion of the chromogen per mouse per 24 hours.

‡ The survival is expressed as the number of mice alive over the number at the beginning.

Other Amino Acids—In a search for other amino acids that might affect the survival of the pyridoxine-deficient mouse, weanling mice partially depleted of the vitamin were divided into groups of four and fed 10 or 60 per cent of casein or 10 per cent of casein plus either 0.95 per cent of *l*-histidine or 2.6 per cent of *l*-tyrosine for 8 days. As usual the mice receiving 60 per cent of casein excreted 500 γ of the chromogen per gm. of food, while those receiving 10 per cent of casein excreted only 30 γ per gm. Histidine and tyrosine failed to increase the excretion of the chromogen. On the 60 per cent casein diet the mice lost an average of 1.9 gm. in 8 days, while those receiving 10 per cent of casein alone or supplemented with either of the amino acids gained 0.1 to 0.7 gm. in the same period. In other words histidine and tyrosine did not appear to be deleterious to the pyridoxine-deficient mouse.

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[8] Schweigert, B. S., Tatman, I., and Elvehjem, C. A., personal communication.

In a similar manner mice were fed 10 per cent of casein plus 1.3 per cent of *dl*-phenylalanine. In the absence of pyridoxine mice receiving phenylalanine were comparable in health for at least 10 days to similar mice not receiving phenylalanine The mice receiving the phenylalanine excreted large quantities of a urinary chromogen which was roughly similar to xanthurenic acid in solubility and in its ability to form a green color with ferric chloride. Significantly, however, the excretion of the phenylalanine chromogen was not prevented by feeding 10 γ of pyridoxine per gm. of diet. The latter chromogen also differed from that excreted by mice fed tryptophane in that the iron complex of the phenylalanine chromogen was unstable in absolute ethyl alcohol. The complex had an absorption spectrum similar to that formed with phenylpyruvic acid[9] and it faded at a comparable rate Both the phenylalanine chromogen and phenylpyruvic acid were detected only in aqueous solutions of pH 2 to 5, rather than at the pH of 7 to 9 used for xanthurenic acid. Calculated as phenylpyruvic acid, the chromogen accounted for 4 to 6 per cent of the phenylalanine ingested in the previous 24 hours. Since both phenylpyruvic (23) and *p*-hydroxyphenylpyruvic (24) acids have been reported to occur in urine as abnormal metabolites of phenylalanine, it is probable that one or both of these were excreted by our mice.

Incidentally, the administration of 100 γ of ascorbic acid and 10 γ of pyridoxine per gm. of diet had no effect on the excretion of this chromogen. The administration of ascorbic acid to certain premature infants has been reported to stop the excretion of *p*-hydroxyphenylpyruvic acid after the administration of phenylalanine or tyrosine (25).

Experiments with Rats—Casein also appeared to be deleterious to the pyridoxine-deficient rat, but less so than to the deficient mouse. In one preliminary series weanling Sprague-Dawley rats receiving 60 per cent of casein and no pyridoxine died in an average of 12 weeks while those on a 12 per cent casein diet lived for 20 weeks. A high casein intake also appeared to hasten death in a second series, but the difference between the two groups was not great. Weanling rats receiving 60 per cent of casein and no pyridoxine excreted 100 to 200 γ of the chromogen per gm. of food after 1 to 2 weeks, and by 4 weeks the excretion rose to 400 or 500 γ per gm. of diet. Similar rats receiving 20 per cent of casein and no pyridoxine excreted less than 100 γ of xanthurenic acid after 4 weeks and 100 to 200 γ per gm. of diet after 16 weeks. In contrast, mice excrete 400 γ of xanthurenic acid per gm. of the high casein diet within 10 days, and on 20 per cent of casein an excretion of 300 γ is reached within 3 to 4 weeks. Adult rats raised on a synthetic diet containing 18 per cent of casein and 6 γ of pyridoxine per gm., and thereafter deprived of pyridoxine for 9 months, maintained themselves as well on 60 per cent of casein as on 12 per cent. On the other hand, adult mice on a 10 per cent casein diet with no pyridoxine survived 3 to 4 times as long as those receiving 60 per cent of casein.

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[9] The phenylpyruvic acid was prepared by Dr. J. A. Miller according to Erlenmeyer and Arbenz (22).

The greater sensitivity to casein of the deficient mouse as compared to the rat may be an expression of its greater requirement for nutrients per unit of body weight. Each day an adult mouse eats dry diet equivalent to 10 or 15 per cent of its body weight, while the adult rat ingests food equivalent to only 5 to 8 per cent of its body weight. This more rapid metabolic turnover might result in a more rapid rate of destruction of the vitamin in the tissues of the mouse. It is also possible that the tissues of the mouse require a higher minimum concentration of pyridoxine for normal metabolism than the tissues of the rat. Another possibility is that the rat may obtain more pyridoxine by intestinal synthesis than does the mouse. It is probable that the relationship between pyridoxine and dietary casein would have been established sooner if the mouse rather than the rat had been used as the experimental animal. Even in the rat, however, it would now appear desirable to control the protein content of the diet when this species is employed in a bioassay for pyridoxine.

SUMMARY

1. A method is described for the estimation of xanthurenic acid in urine. Mice deficient in pyridoxine excreted this chromogen in amounts that depended upon the casein or tryptophane content of the diet.

2. Pyridoxine-deficient mice fed diets containing 60 per cent of casein lived only one-third as long as those fed 10 per cent of casein. Although pyridoxine restored growth and minimized the excretion of chromogen on both diets, about 3 times as much pyridoxine was required on 60 per cent of casein as when 20 per cent was fed.

3. Increases in the excretion of chromogen were quantitatively similar whether *l*-tryptophane was fed as the amino acid or as a component of casein. Calculated as xanthurenic acid the urinary chromogen averaged 10 to 24 per cent of the tryptophane ingested.

4. *l*-Tryptophane decreased the survival time of mice deficient in pyridoxine, but not so much as casein of equivalent tryptophane content. Apparently, therefore, amino acids other than tryptophane also contributed to the ill health of the pyridoxine-deficient mice.

5. Tyrosine or histidine did not affect either the health or the chromogen excretion of deficient mice. The addition of phenylalanine gave rise to a different chromogen, probably *p*-hydroxyphenylpyruvic or phenylpyruvic acid. The excretion of this chromogen was not affected by the administration of pyridoxine or ascorbic acid. Phenylalanine did not appear to interfere with the growth or survival of the deficient mouse.

6. Weanling rats on a 60 per cent casein diet deficient in pyridoxine excreted more xanthurenic acid and died somewhat earlier than rats consuming 20 per cent of casein. However, the differences were much less striking than with mice.

BIBLIOGRAPHY

1. Musajo, L., *Atti accad. Lincei*, **21**, 368 (1935).
2. Musajo, L., and Minchilli, M., *Ber. chem. Ges.*, **74 B**, 1839 (1941).
3. Musajo, L., and Chiancone, F. M., *Atti accad. Lincei*, **21**, 468 (1935).
4. Musajo, L., and Chiancone, F. M., *Gazz. chim. ital.*, **67**, 218 (1937).
5. Lepkovsky, S., and Nielsen, E., *J. Biol. Chem.*, **144**, 135 (1942).
6. Fouts, P. J., and Lepkovsky, S., *Proc. Soc. Exp. Biol. and Med.*, **50**, 221 (1942).
7. Wintrobe, M. M., Follis, R. H., Jr., Miller, M. H., Stein, H. J., Alcayaga, R., Humphreys, S., Suksta, A., and Cartwright, G. E., *Bull. Johns Hopkins Hosp.*, **72**, 1 (1943).
8. Lepkovsky, S., Roboz, E., and Haagen-Smit, A. J., *J. Biol. Chem.*, **149**, 195 (1943).
9. Reid, D. F., Lepkovsky, S., Bonner, D., and Tatum, E. L., *J. Biol. Chem.*, **155**, 299 (1944).
10. Cerecedo, L. R., and Foy, J. R., *Federation Proc.*, **3**, 55 (1944).
11. Foy, J. R., and Cerecedo, L. R., Abstracts, Division of Biological Chemistry, American Chemical Society, Atlantic City (1941).
12. Conger, T. W., and Elvehjem, C. A., *J. Biol. Chem.*, **138**, 555 (1941).
13. Fishman, W. H., and Artom, C., *Federation Proc.*, **3**, 10 (1944).
14. Bellamy, W. D., and Gunsalus, I. C., *J. Bact.*, **46**, 573 (1943).
15. Gunsalus, I. C., and Bellamy, W. D., *J. Biol. Chem.*, **155**, 357 (1944).
16. Atkin, L., Schultz, A. S., Williams, W. L., and Frey, C. N., *Ind. and Eng. Chem., Anal. Ed.*, **15**, 141 (1943).
17. Wesson, L. G., *Science*, **75**, 334 (1932).
18. Cohn, E. J., and Edsall, J. T., Proteins, amino acids and peptides, New York, 358 (1943).
19. Schmidt, C. L. A., The chemistry of the amino acids and proteins, Baltimore, 217 (1938).
20. Block, R. J., and Bolling, D., The determination of the amino acids, Minneapolis, p. iii (1940).
21. Greene, R. D., and Black, A., *J. Biol. Chem.*, **155**, 1 (1944).
22. Erlenmeyer, E., Jr., and Arbenz, E., *Ann. Chem.*, **333**, 328 (1904).
23. Fölling, A., *Z. physiol. Chem.*, **227**, 169 (1934).
24. Levine, S. Z., Marples, E., and Gordon, H. H., *J. Clin. Invest.*, **20**, 199 (1941).
25. Levine, S. Z., Gordon, H. H., and Marples, E., *J. Clin. Invest.*, **20**, 209 (1941).

SPECTROPHOTOMETRIC STUDIES

XIII. THE ABSORPTION SPECTRUM OF ISOLATED DIHYDROCOZYMASE*

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In this communication data will be presented upon the absorption spectrum of isolated dihydrocozymase. As far as the writer is aware, the spectrum has not been reported heretofore as obtained from solutions of the isolated reduced coenzyme. Ohlmeyer (1), who first prepared pure dihydrocozymase, gives only the value for the absorption maximum at wavelength 340 mμ (Table I). Warburg and Christian (2) originally published the absorption spectrum curve of dihydrocozymase, and their curve has been reproduced often in the literature (3). This spectrum, however, was obtained not from the isolated reduced coenzyme but from cozymase, reduced in a 1 per cent bicarbonate medium (pH 9.7) by means of dithionite, $Na_2S_2O_4$, and the mixture then saturated with pure oxygen (2). The treatment with oxygen in the alkaline solution was carried out to inactivate oxidants produced from the $Na_2S_2O_4$. The value of the absorption maximum at 340 mμ in Warburg and Christian's original measurements (2) was approximately 24 per cent lower than that obtained by Ohlmeyer (1) upon isolated dihydrocozymase. However, progressively higher values have been reported in later communications (4–7) from Warburg's laboratory (Table I). It will be pointed out that our values for the extinction coefficient at 340 mμ of isolated dihydrocozymase closely approach the value reported by Ohlmeyer (1), whereas the corresponding values obtained from cozymase reduced by Warburg and Christian's method (2) or enzymically in the presence of arsenate are consistently lower. This discrepancy was disclosed in the course of the study by Meyerhof and the writer of the phosphorylation and oxidation of *d*-glyceraldehyde 3-phosphate, reported in the following paper (8). Its existence is of concern in the quantitative estimation of the concentration of active cozymase present, since in enzymic studies pure cozymase is usually not available.

Methods

Spectrophotometry—The apparatus employed was a photoelectric ultraviolet spectrophotometer, designed by the writer, and described in preliminary reports (9). During several years the apparatus, consisting of a hydrogen discharge tube source, quartz monochromator, powerful photoelectric amplifier, and relatively rugged galvanometer, has afforded unusually stable operating conditions, as demonstrated by the excellent reproducibility of periodic checks upon such standards as K_2CrO_4 in 0.05 N KOH. A detailed description of the equipment and tests of its performance will be published elsewhere.[1] While the spectrophotometer has been designed for adaptability to several alternative photometric procedures (9), in the work reported here and in the following communication (8) the *substitution method* of measurement was employed. In this technique a mechanical switching device, which holds the usual closed type, 1 cm. depth solvent and solution cuvettes provided with quartz end-plates, is used for rapidly substituting one cuvette for the other in a single monochromatic path. The galvanometer deflection, assuming direct proportionality of response to intensity of transmitted radiation, is utilized in obtaining on the scale of 0 to 100 the *fraction of transmitted flux*, I/I_0, where I and I_0 represent respectively the intensities of radiation through the solution and solvent, with I_0 taken as 100 per cent or unity. The extinction or optical density, D, equals $-\log I/I_0$. Since the unit depth of 1 cm. is employed, the value ϵ (c = 1 mM per liter, d = 1 cm.) = D/(concentration (mM per liter) of the solution measured).[2] This simple, rapid photometric procedure represents one of the advantages of the photoelectric technique, and is particularly suitable for studying solutions undergoing change, as in the investigation of enzymic equilibria (8).

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* The construction of the photoelectric ultraviolet spectrophotometer used in this work was made possible through a generous contribution of funds by the Harrison Department of Surgical Research, School of Medicine, University of Pennsylvania.

In the measurements upon isolated dihydrocozymase, distilled water was used as a blank. In calculating the ϵ values from the optical density, the purity of the sample was used as a correction factor for ϵ values at wave-lengths of 375 to 305 mμ. ϵ values at all other wave-lengths, as may be seen in Fig. 1, are uncorrected and related to the concentration based on the original weight of the sample. Purity was determined both manometrically with ferricyanide (4) and by direct titration with iodine The latter procedure is presented in the accompanying paper (8).

In the study with Dr. Meyerhof the amount of dihydrocozymase formed was used as the criterion of the enzymic oxidation and phosphorylation of glyceraldehyde phosphate (8). All solutions were made up in double distilled water and filtered through sintered glass filters (Schott, size F). The oxidizing enzyme was added just before filling the cuvettes, so that measurements could be begun 3 to 5 minutes after mixing. Readings were limited to the chosen wave-lengths of 410 and 340 mμ and were taken at approximately 0.5 to 1 minute intervals, permitting the point of equilibrium (maximum reduction of the cozymase under the particular conditions employed) to be established. It was assumed that the optical density at 410 mμ is zero for dihydrocozymase. The difference at this wave-length between the blank, which contained all the participants of the reaction except cozymase, and the reaction sample was ascribed to unavoidable scattering of light by shreds and other particles and was used for correcting the extinction values at 340 mμ. The concentration, c, of dihydrocozymase was calculated from the corrected extinction values, with the relationship $c = D/\epsilon$ where $\epsilon = 6.27$ (Ohlmeyer's value upon isolated dihydrocozymase (1)). This ϵ value was adopted as a constant, since it appears that Ohlmeyer, working in Meyerhof's laboratory, had in his hands a preparation of the highest purity thus far attained.

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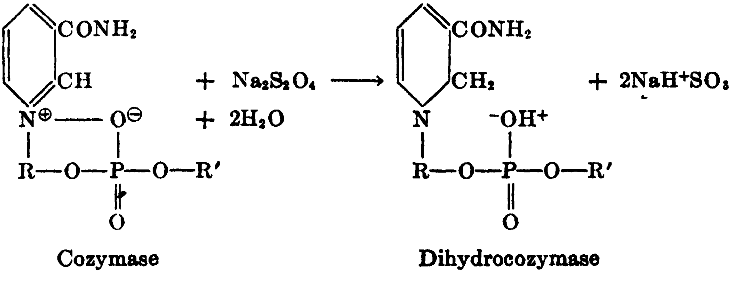
[1] In press, *J. Optical Soc. Am.*

[2] In the notation ϵ (c = 1 mM per liter, d = 1 cm.), $\epsilon = (1/(c \times d)) \times \log I_0/I$, where the concentration c is expressed in mM per liter, the depth d in cm., the intensity of incident light (passing through solvent alone) I_0 is 1.0, and the intensity of transmitted light (passing through the solution) I is expressed as a fraction of unity. In this manuscript the symbol ϵ, defined as above, will be used. By definition our values are equivalent to molecular extinction coefficients $\times 10^{-3}$.

In the case of mixtures of dihydrocozymase and cozymase (present at equilibrium (8)) the concentration not only of the reduced form (determined as above) but also of the oxidized must be estimated. Cozymase can be derived by difference if the concentration of total active coenzyme is known. The latter must usually be determined, since elementary analysis cannot serve as an index of purity in terms of activity of coenzyme preparations. Reference need be made here to only two of the methods available for the estimation of total active coenzyme. In the method adopted by Meyerhof and the writer (8) total active cozymase is determined spectrophotometrically as dihydrocozymase by assuming that the reaction given in Equation 1 goes to completion. In the second procedure total active cozymase is estimated manometrically (2) in a bicarbonate medium in the reaction with $Na_2S_2O_4$, 3 moles of CO_2 (equivalent to $3H^+$) being produced per mole of cozymase reduced. This reaction may be written as in Equation 2. We have failed to obtain agreement by these two

$$d\text{-Glyceraldehyde 3-phosphate} + \text{cozymase} \xrightarrow{\textit{arsenate, oxidizing enzyme}} d\text{-glyceric acid 3-phosphate} + \textit{dihydrocozymase} \qquad (1)$$



(2)

analytical procedures for total active cozymase, and have consistently found 12 to 15 per cent lower values in the spectrophotometric estimation (enzymic reaction with arsenate) than those yielded by manometry with dithionite. The explanation of this discrepancy is not clear. A deviation in the same direction from the results of the manometric technique was obtained also when the production of dihydrocozymase by means of $Na_2S_2O_4$ was determined spectrophotometrically from the extinction values at 340 mμ, Ohlmeyer's constant (1) and Warburg and Christian's method (2) of stabilization after reduction by saturation with oxygen at pH 9.7 being applied. In these spectrophotometric measurements successive readings indicated that the reoxidation of dihydrocozymase was not completely prevented in the dithionite procedure.

Preparations Used—The dihydrocozymase was an air-dried preparation, isolated as the disodium salt (mol. wt 709) by Ohlmeyer's method (1). The adenylic acid (mol. wt. 347) was prepared from muscle by the method of Lohmann (10).

Results

The absorption spectrum curve of isolated dihydrocozymase is given in Fig. 1. For purposes of comparison, the curve obtained from pure, crystalline adenylic acid is included

In Table I ϵ values are collected both from the literature and the present work for the characteristic maximum of dihydrocozymase at 340 mμ In Column 8 ϵ values are listed in terms of the percentage of Ohlmeyer's value of $\epsilon = 6.27$, used as a standard of comparison Attention may be called to the following (1) On the basis of our own comparative measurements ϵ values (corrected for purity, Column 7, Table I) obtained from isolated dihydrocozymase (Analyses 11 and 12) approached closely the value reported by Ohlmeyer (Analysis 9), and were consistently and significantly higher than the ϵ values (Analyses 7 and 8) for dihydrocozymase produced from cozymase in solution by Warburg and Christian's method of reduction (2), or enzymically in the presence of arsenate (8) (2) The agreement of independent spectrophotometric techniques was satisfactory. Analyses 6, 7, and 8 (Table I) were performed upon aliquots of the same preparation[3] The average of our ϵ values (Analyses 7 and 8) is 5 46, a figure practically identical with that obtained by Schlenk (Analysis 6) who employed a quartz spectrograph with sector photometer (11). (3) The purity of the preparation must be determined accurately Analysis 10, Table I, suggests that less reliance may be placed upon the apozymase test (12) for the purity of dihydrocozymase than upon the ferricyanide (Analyses 11a and 12a) or iodine (Analyses 11b and 12b) reactions. (4) In work upon isolated dihydrocozymase a pitfall, illustrated in Analysis 13, Table I, must be avoided. In Ohlmeyer's isolation procedure (1) cozymase is not only reduced, but purification is accomplished. In this particular experiment the procedure did not have a successful outcome. It was applied to a very small quantity of cozymase, an aliquot of Schlenk's preparation (Analyses 6, 7, and 8). The purity (Column 4) of the isolated product proved to be lower than the original and the final solution had a yellowish color, both suggestive of the formation of monohydrocozymase. The low ε value at 340 mμ is in accord with the probability that the isolated product was a mixture of monohydro- and dihydrocozymase.

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[3] A sample of this preparation (11) was very kindly supplied by Dr F Schlenk, School of Medicine, University of Texas

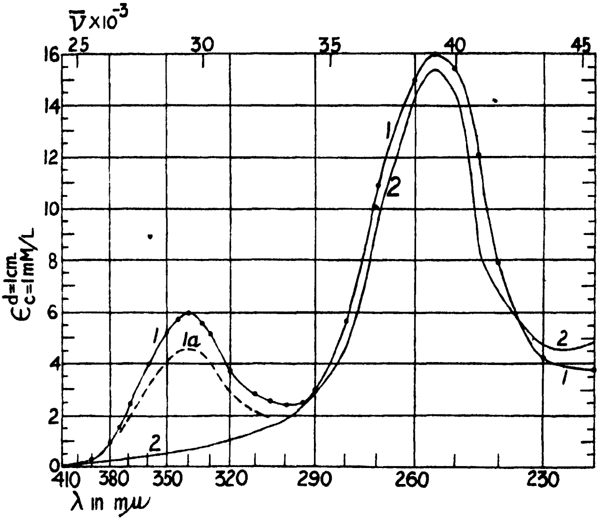

FIG 1. The absorption spectrum curves of isolated dihydrocozymase and adenylic acid. Curve 1, average curve based on two sets of measurements on the same solution, carried out 6 days apart with the solution stored in a refrigerator during the interval 2 85 mg of disodium dihydrocozymase (mol. wt. 709) were dissolved in 25.5 ml. of double distilled water, diluted 1:3 before measurement. The concentration based on the weight of the sample = 0.0525 mM per liter. The purity based on iodine titration (8) was for the two runs respectively 77.7 and 72.0 per cent. Correction for purity was applied only in the region 375 to 305 mμ (see the text). The broken line (Curve 1a) indicates the result when correction for purity is not applied. The blank was double distilled water, and transmission for the blank at 410 mμ was taken arbitrarily as 100 per cent. Curve 2, 13.5 mg. (equivalent to 13.15 mg. of dry weight) of pure, crystalline adenylic acid (mol. wt. 347) per liter of double distilled water. The concentration = 0.0378 mM per liter. For the blank see the legend of Curve 1.

DISCUSSION

An explanation is in order in justification of the arbitrary limitation of the correction for purity to the ε values of dihydrocozymase at wave-lengths of 375 to 305 mμ. This is the spectral area covered by the specific absorption band with a maximum at 340 mμ. Cozymase has no band in this region. Both the reduced and oxidized forms of the coenzyme have the

TABLE I

Comparison of Our ε Values with Those Reported in Literature for Characteristic Maximum at 340 Mμ of Dihydrocozymase

Analysis No. (1)	Type of preparation* (2)	Author (3)	Purity (4)	Concentration of dissolved substance (5)	Concentration of active substance (6)	ε† (7)	Per cent of Ohlmeyer's ε value (8)
			per cent	*mM per l*	*mM per l*		*per cent*
1	A	Warburg and Christian (2)	‡			4.78§	76.2
2	"	Haas (4)	‡			5.90§	94.1
3	"	Negelein and Wulff (5)	‡			5.12§	81.7
4	"	" " Bromel (6)	‡			5.47§	87.2
5	"	Kubowitz and Ott (7)	‡			6.27§	100.0
6	"‖	Schlenk (11)	85.0¶			5.50	87.7
7	"‖	Present observer	80.2**	0.0511	0.0410	5.27	84.0
8	"‖	" "	68.6**	0.0977	0.0670	5.66	90.2
9	B	Ohlmeyer (1)	95–100††			6.27§	
10	"	Present observer	83.0††	0.0647	0.0537	5.49	87.6
11a	"	" "	78.2‡‡	0.0525	0.0410	5.76	91.8
11b	"	" "	77.7§§	0.0525	0.0407	5.80	92.5
12a	"	" "	74.2‡‡	0.0525	0.0389	5.96	95.0
12b	"	" "	72.0§§	0.0525	0.0378	6.14	97.8
13	"‖ ‖	" "	68.0§§	0.1185	0.0806	3.92	62.5

* Preparations A, cozymase reduced in bicarbonate solution with $Na_2S_2O_4$ (2), Preparations B, dihydrocozymase isolated by Ohlmeyer's method (1)

† ε, corrected for purity in those cases in which the data for purity (Columns 4 and 6) are furnished. For notation see foot-note 2.

‡ Preparation considered "pure" by respective authors. Kubowitz and Ott (7) use ε = 6.27 in the text of their paper. It is assumed that they have experimentally verified Ohlmeyer's value.

§ Calculated from β coefficients, with the relationship $\epsilon = 0.4343 \times (\beta \times 10^{-6})$

‖ Samples from same original preparation of cozymase

¶ Purity based on phosphorus content of 8.0 per cent.

** Purity determined manometrically with $Na_2S_2O_4$ (2)

†† Purity based on apozymase test (12). Ohlmeyer (1) used both the apozymase test and the phosphorus content for purity.

‡‡ Purity based on ferricyanide-manometric procedure (4)

§§ Purity based on direct titration with iodine (8).

‖ ‖ Prepared from aliquot of Schlenk's cozymase (Analysis 6) See the text.

"non-specific" absorption band with a maximum in the region 255 mμ. The latter band may be ascribed to the presence of adenine in the structure. It is evident from Fig. 1 that adenylic acid also has absorption in this spectral region, with an ε value at the maximum of a magnitude similar to the corresponding band of dihydrocozymase. Our ε values at 255 mμ are for dihydrocozymase and pure adenylic acid respectively 15.9 and 15.1. Although no correction for purity has been applied to the absorption in this spectral region in the case of dihydrocozymase, both ε values are somewhat higher than corresponding values for this maximum recorded in the literature (for dihydrocozymase (2), and for adenylic acid (13)). It may also be pointed out that for preparations of dihydrocozymase of different purity (Column 4, Table I) appreciably smaller deviations were obtained for the ε values at 255 mμ than for the specific maximum at 340 mμ. An example is the poor preparation (Analysis 13, Table I), for which the uncorrected ε value at 340 mμ is 2.66, or only 43 per cent of the adopted standard value of 6.27, while at 255 mμ, uncorrected, ε = 14.2, or 89 per cent of the value of 15.9.

In elucidating the phenomena of absorption it is of interest to inquire as to what change in structure may be held responsible for the appearance of the 340 mμ band in the spectrum of dihydrocozymase. Upon reduction, the aromatic pyridine ring of cozymase is changed to a structure with conjugated double bond character (see Equation 2). The origin of the new band may very probably be ascribed to the formation of the conjugated double bond system. This view is supported experimentally in the study by Meyerhof, Ohlmeyer, and Möhle (14) of the cozymase-bisulfite addition product In this compound, without reduction, the aromatic character of the pyridine ring is lost, and a new band appears in the region of 320 mμ. Karrer and associates (15) have shown that nicotinamide iodomethylate, like the coenzyme, upon reduction with $Na_2S_2O_4$ yields an analogous spectrum, with a new band at 360 mμ.

In the spectrophotometric estimation of dihydrocozymase, Ohlmeyer's ε value of 6.27 (1) has been adopted (Drabkin and Meyerhof (8)) In the present measurements on isolated dihydrocozymase, with purity based on the ferricyanide (4) or iodine (8) procedures, this value was approached very closely. However, consistently lower ε values were obtained on cozymase reduced in solution by $Na_2S_2O_4$, with purity determined by the dithionite-manometric technique (2). Hence it is obvious that in our hands (8) the spectrophotometric estimation of active cozymase, with ε = 6.27, yields lower values than those obtained by manometry.

The writer is indebted to Dr. Otto Meyerhof for cooperating in this work and for furnishing the sample of pure adenylic acid and preparations of dihydrocozymase.

SUMMARY

1. The absorption spectrum of isolated dihydrocozymase has been measured.

2. A comparison has been made of the ε values for the maximum of the specific band of the reduced coenzyme at 340 mμ, obtained from isolated dihydrocozymase and from cozymase reduced in a bicarbonate medium with $Na_2S_2O_4$. The implications of the measurements have been discussed. The origin of the 340 mμ band may be related to the change from the aromatic pyridine ring in cozymase to a structure possessing conjugated double bond character in the reduced coenzyme.

BIBLIOGRAPHY

1. Ohlmeyer, P., *Biochem Z.*, **297**, 66 (1938)
2. Warburg, O , and Christian, W , *Biochem Z* , **287**, 291 (1936).
3. Schlenk, F., in A symposium on respiratory enzymes, Madison, 104 (1942).
4. Haas, E., *Biochem. Z.*, **291**, 79 (1937).
5. Negelein, E., and Wulff, H. J., *Biochem Z.*, **293**, 351 (1937).
6. Negelein, E., and Brömel, H , *Biochem. Z* , **301**, 135 (1939); **303**, 132 (1939).
7. Kubowitz, F., and Ott, P , *Biochem. Z.*, **314**, 94 (1943).
8. Drabkin, D. L., and Meyerhof, O , *J. Biol. Chem.*, **157**, 571 (1945).
9. Drabkin, D L., Proceedings, 7th summer conference on spectroscopy and its applications, 1939, New York, 85 (1940); in Glasser, O., Medical physics, Chicago, 967 (1944).
10. Lohmann, K , *Biochem. Z* , **233**, 460 (1931).
11. Schlenk, F., *J. Biol. Chem.*, **146**, 619 (1942).
12. Myrbäck, K., in Nord, F. F., and Weidenhagen, R., Ergebnisse der Enzymforschung, Leipzig, **2**, 139 (1933). von Euler, H. *Ergeb. Physiol.*, **38**, 1 (1936). Ohlmeyer, P., *Biochem. Z.*, **287**, 212 (1936)
13. Gulland, J. M., and Holiday, E. R , *J. Chem Soc.*, 765 (1936). Kalckar, H. M., *Science*, **99**, 131 (1944).
14. Meyerhof, O., Ohlmeyer, P., and Mohle, W., *Biochem. Z.*, **297**, 113 (1938).
15. Karrer, P., Schwarzenbach, G., Benz, F., and Solmssen, U., *Helv. chim. acta*, **19**, 811 (1936). Karrer, P., and Warburg, O., *Biochem. Z.*, **285**, 297 (1936).

A SPECTROPHOTOMETRIC STUDY OF THE OXIDATION AND PHOSPHORYLATION OF *d*-GLYCERALDEHYDE 3-PHOSPHATE*

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The oxidative step of alcoholic fermentation may be written[1]

$$d\text{-Glyceraldehyde 3-phosphate} + \text{phosphate} + \text{cozymase} \rightleftharpoons d\text{-glyceric acid 1,3-diphosphate} + \text{dihydrocozymase} \quad (1)$$

The thermodynamic aspect of the reaction appears clear and important (1). The process provides and example, thus far unique, of the direct (rather than indirect (2)) coupling of an oxidation with the creation of an energy-rich phosphate bond. The reaction may be viewed as *the energy donator which initiates the transphosphorylation process* requiring at all subsequent steps the participation of transphosphorylase and adenosine triphosphate.

Although the 2-fold character of the above process, oxidation and phosphorylation, is evident, the mechanism of the phosphorylation still requires clarification. Warburg and Christian (3) and Negelein and Brömel (4) have postulated upon purely theoretical grounds the formation of an intermediate chemical entity, glyceraldehyde 1,3-diphosphate, whose production was assumed to be wholly a function of the concentration of phosphate. By determining "total" glyceraldehyde phosphate (monophosphate + possible diphosphate), Meyerhof and Junowicz-Kocholaty (5) failed to demonstrate any effect of the addition of phosphate alone or together with cozymase and the oxidizing enzyme (3) upon the equilibrium between glyceraldehyde phosphate and dihydroxyacetone phosphate (isomerase). They reached the conclusion that phosphorylation in the chemical sense is accomplished only in the formation of the glyceric acid 1,3-diphosphate, as a direct result of the oxidative process.

The work to be reported was undertaken as a supplement of the latter chemical studies. The equilibrium represented in Equation 1 and related equilibria were investigated by means of the spectrophotometric determination of the reduction of cozymase. Under certain conditions, which will

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* This work was aided by grants to one of us (O M) from Hoffmann-La Roche, Inc., Nutley, New Jersey, and from the Penrose Fund of the American Philosophical Society. The construction of the spectrophotometer used in the measurements was made possible by funds furnished by the Harrison Department of Surgical Research. School of Medicine, University of Pennsylvania.

[1] The term phosphate represents inorganic phosphate throughout

be described, our data suggest that an unstable addition product, a precursor of the glyceric acid diphosphate, may indeed be formed from glyceraldehyde monophosphate. As far as we know, this is the first positive evidence for a precursor which has been obtained.

As has been indicated in a preliminary statement (6) and note (7) by one of us (O. M.), the dimeric glyceraldehyde diphosphate[2] of Baer and Fischer (7) has proved to be biologically inactive. This synthetic triose diphosphate therefore is not the sought for precursor of glyceric acid 1,3-diphosphate. However, the dimeride can be hydrolyzed into monomeric glyceraldehyde 3-phosphate (Fischer-Baer ester (8)) and phosphate by heating for some minutes in dilute HCl. This reaction is in itself evidence for the structure of the diester as given by the Toronto workers (7). Some data upon equilibrium measurements (Equation 1) in which the dimeric diphosphate, before and after its hydrolysis, was substituted for glyceraldehyde 3-phosphate will be included in this paper.

During the course of the work it has been found that dihydrocozymase can be titrated easily with iodine in neutral solution. This determination should prove useful as a measure of the purity of the reduced coenzyme. The determination is more simple and direct, and just as sensitive as Haas' manometric titration with potassium ferricyanide (9).

Theory and Comments

The equilibrium, which holds for Equation 1, is independent of the manner (chemical union or physical association) in which phosphate may combine with the triose monophosphate, and may be represented by

$$K = \frac{[\text{glyceric acid diphosphate}] \times [\text{dihydrocozymase}]}{[\text{glyceraldehyde phosphate}] \times [\text{phosphate}] \times [\text{cozymase}]} \tag{2}$$

In Equation 2 and those which follow K represents $K_{\text{oxidation}}$. Since, under our conditions, glyceric acid diphosphate undergoes no further change, its molecular concentration must be equal to that of dihydrocozymase. Hence

$$\frac{[\text{Dihydrocozymase}]^2}{[\text{Cozymase}]} = K \times [\text{glyceraldehyde phosphate}] \times [\text{phosphate}] \tag{3}$$

Notation—Let [dihydrocozymase]^2/[cozymase] = r, and let the equilibrium concentrations of glyceraldehyde phosphate and phosphate be represented by g' and p' respectively, and their initial concentrations by g and p. Now, in the absence of the hypothetical addition product, which may be symbolized by a, Equation 3 may be written

$$\frac{r}{K} = g' \times p' \tag{4}$$

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[2] Kindly supplied by Dr. Hermann O. L. Fischer of the Banting Institute, Toronto.

The equilibrium concentrations of g' and p' here would be equal to their initial concentrations g and p minus the amount transformed into glyceric acid diphosphate, which is taken equal to dihydrocozymase (Equation 2). If the equilibrium concentration of dihydrocozymase is represented by h,

$$\frac{r}{K} = [g - h] \times [p - h] \tag{5}$$

Equation 5 is equivalent to Equation 4, since $g' = g - h$ and $p' = p - h$, and this relationship should apply in the absence of formation of the addition product, a. However, if the latter is formed in measurable amount, Equation 5 must be modified to include a, yielding

$$\frac{r}{K} = [g - (h + a)] \times [p - (h + a)] \tag{6}$$

In the graphical presentation of the dependence of r upon the concentrations of glyceraldehyde phosphate and phosphate, Equation 6 must lead to a different curve than that expected from Equation 5 or its equivalent, Equation 4, especially when p is much larger than g. Because of the range of concentrations employed, it was convenient to use the logarithmic form of Equation 4; namely,

$$\log r = \log g' + \log p' + \log K \tag{7}$$

When p is kept at a constant level, at least 10 times higher than g (Table III), which is varied, p' may be assumed to be practically equal to p and constant. Hence p' and K may be collected into a single constant, K', and Equation 7 may be changed to

$$\log r = \log g' + \log K' \tag{8}$$

If this relationship applies, log r plotted against log g' should yield a straight line of slope 1.0 or 45°. It is evident from Fig. 1 that the data, obtained under the above conditions, fit this relationship. Therefore, for varying concentrations of glyceraldehyde phosphate, simple mass law assumptions appear applicable.

The situation is more complex when p is varied and g kept constant. Under the actual experimental conditions employed, an approach to constancy of g' at a moderately low level was obtained (Table III). Some deviation is unavoidable, however, since when g is low the relative magnitude of h is significant ($g' = g - h$, Equation 5). Under these conditions Equation 7 applies, and the direction of deviation is such that a plot of log r against log p' may be expected theoretically to yield a line with a slope somewhat less than unity (less than 45°). Our data (Fig. 2) show a consistent deviation in the opposite direction. Log r varies linearly with log p', but the slope of the lines is close to 60°; *i.e.*, approaches a slope of 2.0. Thus, for changing concentrations of phosphate the equilibrium is more

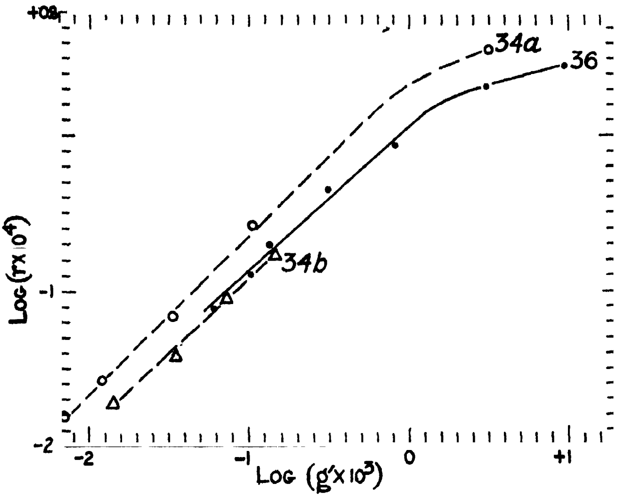

FIG. 1. The relationship at equilibrium of log (dihydrocozymase[2])/(cozymase) and log (glyceraldehyde phosphate). The triose phosphate concentration was varied while the inorganic phosphate concentration was kept constant and relatively high.

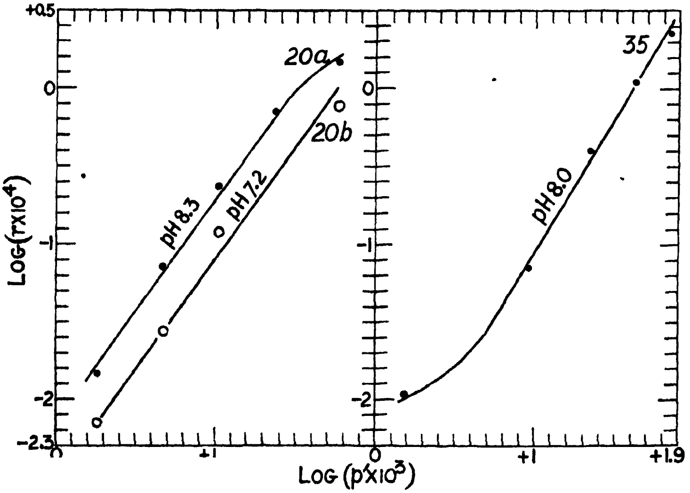

FIG. 2. The relationship at equilibrium of log (dihydrocozymase[2])/(cozymase) and log (inorganic phosphate). The inorganic phosphate concentration was varied and the triose phosphate concentration maintained relatively constant.

complicated than for variations in glyceraldehyde phosphate, and simple mass law formulations are inapplicable. Apparently phosphate has another influence upon the equilibrium besides the final formation of the glyceric acid diphosphate or the formation of a diphosphate precursor (a). If a had been formed in sufficient quantity to be an appreciable fraction of p and g, values could be expected (Equation 6) which would fall appreciably below, rather than above the theoretical slope of 45°.

The negative nature and the limitations of the above approach to the demonstration of or disproof of the existence of a hypothetical precursor are evident. A further approach to a solution of the problem is possible. The formation of dihydrocozymase in a system such as that described above may be compared with its production in a modified system in which the glyceraldehyde phosphate has been replaced by hexose diphosphate and zymohexase. In the latter case the system is kept supplied with the triose monophosphate through the action of zymohexase upon hexose diphosphate. At equilibrium in this reaction it has been established (5) that 4.2 per cent of the total triose phosphate produced is glyceraldehyde phosphate. Hence, the amount of the latter may be calculated, and the amount of dihydrocozymase produced should correspond to this quantity, provided no intermediary or addition product is formed.

If appreciable amounts of the addition product are formed in this case, more glyceraldehyde phosphate will be produced from the hexose diphosphate and more dihydrocozymase than calculated for 4.2 per cent of aldotriose in the zymohexase equilibrium may be expected. Actually, this very situation was encountered in certain experiments with high concentrations of phosphate and an excess of the oxidizing enzyme. The amount of dihydrocozymase produced was 2- to 3-fold greater than in the corresponding experiments with glyceraldehyde phosphate (protocols of Experiments 36a and 39).. This positive outcome seems to be consistent with the postulation of an intermediate.

Methods

Determination of Dihydrocozymase and of Total Active Cozymase—Spectrophotometry was by the substitution method, described in the preceding communication (10). An ultraviolet photoelectric spectrophotometer, designed by one of us (D. L. D.), was employed. Since both the oxidized and reduced forms of the coenzyme participate in the equilibrium, it was necessary to determine not only the dihydrocozymase, but also, under the same conditions, the total amount of active cozymase present. In the spectrophotometric estimation of each of these quantities Ohlmeyer's value ϵ (c = 1 mM per liter, d = 1 cm.) at wave-length 340 mμ = 6.27[3] was adopted as a constant (10, 11). As pointed out in the preceding paper (10), using the value 6.27 in the determination, we have consistently obtained 12 to 15 per cent lower values for the activity of five different samples of cozymase (reduced by arsenate in the presence of the oxidizing enzyme) than those yielded by manometry in the reaction with $Na_2S_2O_4$ (12). The spectrophotometric evaluation was adopted to secure consistency in both sets of analyses.

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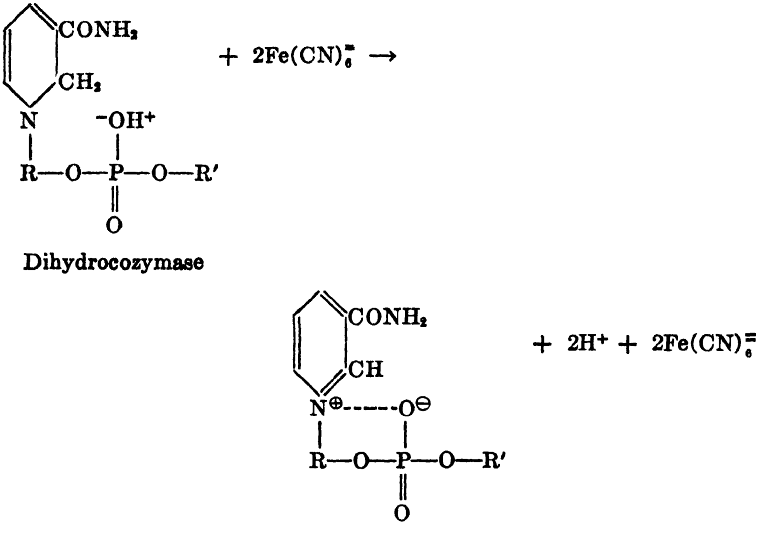
[3] Calculated from Ohlmeyer's β coefficients (11), with the relationship ϵ (c = 1 mM per liter, d = 1 cm.) = $0.4343 \times (\beta \times 10^{-6})$.

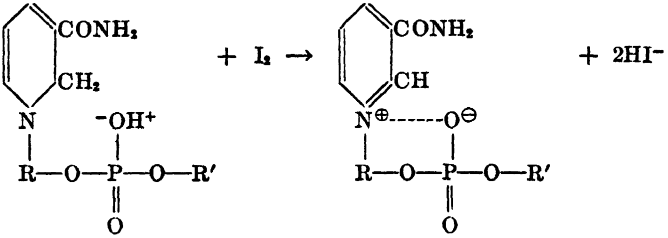
Titration of Dihydrocozymase in Neutral Solution with Iodine—The purity of isolated dihydrocozymase preparations can be measured manometrically by Haas' method (9) of oxidation with ferricyanide in a bicarbonate medium. The reaction may be pictured as given in Equation 9. It is evident that in the reaction $\Delta H^+ = 1$, yielding 1 mole of CO_2 (measured manometrically). A new reaction, which is more simple and direct than the ferricyanide manometry technique, has been carried out. Dihydrocozymase is titrated in neutral solution with iodine, in a micro burette. 2 equivalents of I^- correspond to 1 mole of dihydrocozymase in the reaction shown in Equation 10.



Dihydrocozymase

Cozymase

(9)



(10)

Dihydrocozymase Cozymase

A comparison of the ferricyanide and iodine methods is provided by the data presented in Table I. Spectrophotometry was performed upon the same preparation of dihydrocozymase with the results reported in the preceding paper (10).

The preparation of the different enzymes and substrates has been described by Meyerhof and Junowicz-Kocholaty (5).

EXPERIMENTAL

The amount of oxidizing enzyme necessary for establishing the equilibrium in 5 to 10 minutes varied with the activity, which declined with the age of the preparations. In Table II data are presented to illustrate the order of activity of a rather fresh enzyme preparation (1 week old). Each sample was made up to 3.6 ml., and had the following composition per ml.: cozymase, 0.146 mg., spectrophotometric determination with arsenate = 1.75×10^{-4} M, total triose phosphate, containing 0.094 mg. of alkali-saponifiable P, of which 0.064 mg. of P = 2.07×10^{-3} M glyceraldehyde phosphate, phosphate (pH 8.0) = 42×10^{-3} M, glutathione = 2.8×10^{-3} M; 0.056 ml. of a diluted solution of oxidizing enzyme, prepared from a stock solution containing 7.8 mg. of protein per ml. In Experiment 4, Table II, effective activity was obtained with a concentration of enzyme of approximately 1 part in 7 million.

TABLE I

Comparison of Results by Iodine and by Ferricyanide Methods

Preparation No.	Dihydrocozymase*	0.005 N I_2 titration	Ferricyanide method, CO_2 measured	Concentration $\times 10^3$† dihydrocozymase	Purity	
					Iodine method	Ferricyanide method
	mg.	*ml.*	*c.mm.*	*M*	*per cent*	*per cent*
1	5.90	2.590		6.47	77.7	
1	6.15		152	6.78		78.2
1a‡	2.52	1.055		2.64	74.2	
1a	1.89	0.800		2.00	74.9	
1a	3.78		86	3.84		72.0

* Disodium salt of dihydrocozymase (mol. wt. 709), prepared by Ohlmeyer's method (11) and air-dried.

† Calculated from the analytical data.

‡ Same as Preparation 1, tested 1 week later.

In Table III data from several typical experiments are collected for the equilibrium of Equation 2. In Experiments 36, 34a, and 34b the concentration of glyceraldehyde phosphate was varied, while that of phosphate was kept constant and relatively high (approximately 10 times higher than the highest concentration of the aldotriose). In Experiments 35, 20a, and

20b the condition at equilibrium was explored by varying the concentration of phosphate but maintaining the concentration of the aldotriose relatively constant.

The calculated values in Column 7 of Table III emphasize the difficulties of exact control (due to deterioration of enzymes or other factors) in experiments of this type. It is evident that a reliable value for an equilibrium constant, K, cannot be established on the basis of the present measurements. However, this does not interfere with the interpretation, since it is clear that, except for discrepant values (enclosed in parentheses) at extremes of concentration (in the case of variation in glyceraldehyde at high concentrations of the triose phosphate and with variation in phosphate at low concentrations of the latter), relatively constant values are obtained in individual experiments. There can also be no doubt of the real difference in results in the two sets of experiments, which is reflected in the difference of slope of the plotted relationships. In Fig. 1, with variation in glyceraldehyde phosphate concentration, the values fall along lines of slope 1.0 (or 45°), whereas with variation in phosphate concentration (Fig. 2) the data consistently fit a slope of approximately 2.0 (or 60°). The influence of pH upon this equilibrium (evident in Experiments 20a and 20b) has been disclosed previously (3). The interpretation of these findings has already been presented.

TABLE II

Activity of Typical, Week-Old Preparation of Oxidizing Enzyme

Experiment No.	Enzyme protein added	Time[*] for attaining equilibrium	D[†]	Concentration × 10^4 dihydrocozymase formed[‡]
	γ	*min.*		M
1	22	<10	0.558	0.891
2	4.4	<12	0.548	0.874
3	1.62	16	0.597	0.952
4	0.54	22	0.560	0.893
5	0.22	(20)	(0.442)[§]	
5	0.22	(45)	(0.523)[§]	
Average........			0.566	0.903

[*] Time elapsed after mixing with oxidizing enzyme.

[†] D = extinction, or optical density.

[‡] Concentration of dihydrocozymase calculated from D/ϵ, where $\epsilon = 6.27$ (see the text, under "Methods").

[§] Omitted from the average, since under these conditions equilibrium was evidently not attained.

TABLE III

Data upon Equilibrium (Equation 2)

Experiment No. (1)	Concentration $\times 10^4$ total cozymase* (2)	Concentration $\times 10^4$ dihydrocozymase (3)	$r \times 10^4$† (4)	$g' \times 10^3$† (5)	$p' \times 10^3$† (6)	K‡ (7)
	M	*M*		*M*	*M*	
36	1.94	1.315	2.77	9 32	69	(0.43)
		1.220	2.07	3.03	69	(0.99)
		0.940	0.88	0.805	69	1.59
		0.735	0.45	0.305	69	2.14
		0 530	0.20	0.136	69	2.13
		0.435	0.126	0.105	69	1.74
		0 350	0.077	0.060	69	1.86
34a	1.75	1.285	3.55	3.18	42	(2.66)
		0.555	0.26	0.105	42	5.89
		0.313	0.068	0.033	42	4.90
		0 200	0.026	0.012	42	5.16
		0 153	0 015	0.007	42	5.10
34b§	1 75	0.470	0.173	0.148	42	2.78
		0 360	0 093	0.074	42	2.99
		0 240	0.038	0.036	42	2.51
		0 173	0 019	0 014	42	3.23
35 (pH 8 0)	1 75	1 160	2.28	1.95	69.7	1.68
		0 950	1.13	1 97	42.2	1.36
		0.660	0.40	2.00	21.9	0.89
		0 323	0.073	2.04	9.3	(0.38)
		0 135	0 011	2 05	1.55	(0.35)
20a (" 8 3)	1.87	1.080	1.477	2.84	58.7	0.89
		0 850	0.708	2.87	23.1	1.07
		0.556	0.237	2 90	10.25	0.80
		0.334	0.073	2.92	4.62	(0.54)
		0.160	0.015	2.93	1.78	(0.29)
20b (" 7 2)	1 87	0 870	0.757	2.86	58 7	0.45
		0.424	0.124	2.91	10.25	0.43
		0.210	0.027	2.93	4.63	0.20
		0 108	0.007	2.94	1.79	(0.13)

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* Determined spectrophotometrically, with arsenate.

† The symbols r, g', and p' (explained under "Theory and comments") stand respectively for the ratio (dihydrocozymase^2)/(cozymase), the equilibrium concentration of glyceraldehyde phosphate, and the equilibrium concentration of inorganic phosphate.

‡ Values of the equilibrium constant, calculated by dividing the values in Column 4 by the product of the corresponding values in Columns 5 and 6 For values in parentheses see the text.

§ In this experiment isomerase was employed and the glyceraldehyde phosphate concentration was taken as 4.2 per cent of the total triose phosphate (5).

The data at equilibrium under the above conditions were compared with those obtained in the system hexose diphosphate + zymohexase (see "Theory and comments"). Our problem was to find out what concentration of glyceraldehyde phosphate, in the presence of a constant high concentration of phosphate, would equal that which results from the hexose diphosphate system. To be certain that in the latter case the zymohexase equilibrium had actually been reached in the presence of cozymase and oxidizing enzyme, a third combination, consisting of triose phosphate and zymohexase, was tested also. The same equilibrium distribution could be expected in the triose phosphate and hexose diphosphate systems, provided that the initial concentration of the former was taken equal to the latter in respect to the P content. The following examples show that this was the case.

Protocol of Experiment 36a—For values of g' refer to Experiment 36, Table III. Composition of samples per ml., total cozymase (arsenate value) = 1.94×10^{-4} M, oxidizing enzyme = 14 γ, glutathione = 3×10^{-3} M, zymohexase = 0.083 ml. of a 5 times diluted stock preparation (5), phosphate (pH 8) = 69×10^{-3} M, and besides, in Sample 1, hexose diphosphate in terms of P = 0.364 mg. of P, in Sample 2, triose phosphate in terms of alkali-labile P = 0.41 mg. of P, in Sample 3, a mixture of hexose diphosphate (0.24 mg. of P) and triose phosphate (0.135 mg. of P). The following optical density values, D, were obtained: Sample 1, 0.305, Sample 2, 0.300, and Sample 3, 0.300. The last value of D, corresponding to 0.479×10^{-4} M dihydrocozymase (h), would be obtained in the absence of zymohexase at a concentration of $g' = 0.120 \times 10^{-3}$ M, interpolated from the values in Experiment 36, Table III, where $h = 0.435 \times 10^{-4}$ M corresponds to $g' = 0.105 \times 10^{-3}$ M and $h = 0.53 \times 10^{-4}$ M corresponds to $g' = 0.136 \times 10^{-3}$ M.

For ascertaining the concentration of triose phosphate established by the zymohexase in these experiments, a sample exactly similar to Sample 1, except for the replacement of phosphate by borate of pH 8, was incubated at the same temperature (23°) for 10 minutes and the alkali-labile P determined. Per ml. 47.2 γ of alkali-labile P were found. 4.2 per cent (isomerase equilibrium) of this value, or 1.98 γ of P, may be calculated to represent glyceraldehyde phosphate. 1.98 γ of P per ml. correspond to 0.064×10^{-3} M of g'. The value actually obtained in the above tests was 0.120×10^{-3} M, or twice as great.

Protocol of Experiment 39—The same procedure was used as in Experiment 36a, with nearly the same concentrations for all components, but with phosphate (69×10^{-3} M) of pH 7 instead of pH 8. Sample 1, containing hexose diphosphate (0.358 mg. of P per ml.), gave $D = 0.220$, which corresponds to $h = 0.351 \times 10^{-4}$ M. Sample 2, with triose phosphate (0.375 mg.

of alkali-labile P per ml.), gave $D = 0.256$, corresponding to $h = 0.408 \times 10^{-4}$ M. Sample 3, containing per ml. 0.24 mg. of hexose diphosphate P and 0.125 mg. of triose phosphate P, yielded $D = 0.212$, or $h = 0.338 \times 10^{-4}$ M. In the absence of zymohexase a closely similar value, $D = 0.216$, was obtained from a triose phosphate sample which contained 6.9 γ of glyceraldehyde phosphate P per ml. or 0.222×10^{-3} M. Since h was 0.034×10^{-3} M (from $D = 0.216$), $g' = g - h = 0.188 \times 10^{-3}$ M.

Determination of the triose phosphate in the zymohexase equilibrium, when phosphate is replaced by borate of pH 7.3, gave in 10 minutes at the same temperature (23°) 53 γ of alkali-labile P per ml. 4.2 per cent of this value is 2.23 γ, or 0.072×10^{-3} M. Actually the corresponding value of g' in the above tests (Sample 3) was 0.188×10^{-3} M, or 2.6 times greater.

TABLE IV

Manometric Experiments with Dimeric Glyceraldehyde 1,3-Diphosphate[*]

Substrate	CO_2 produced		
	Experiment 1	Experiment 2	Experiment 3
	c mm	*c mm*	*c mm*
Triose phosphate	21	33	28
Glyceraldehyde diphosphate, direct	0		0
" " after hydrolysis[†]		19.5	
" " with phosphorylase.			0
Hexose diphosphate		0	

[*] In each sample the manometry was performed upon a volume of 1 ml., at 20°, and after incubation for 20 minutes. 1 ml. of the reaction mixture contained 0.1 ml. of active oxidizing enzyme, 0.5 mg. of cozymase, an amount of diester which corresponded to 0.2 mg. of P in terms of *d*-glyceraldehyde 3-phosphate, and had a concentration of 3×10^{-3} M of arsenate.

[†] Heated for 5 minutes at 100° in 0.1 N HCl.

Similar results were obtained in four more experiments of this type, but in some others the values of the zymohexase experiments nearly agreed with those calculated for g'. We believe that in the negative cases the oxidizing enzyme was less active or damaged by the presence of the impure zymohexase solutions. The same explanation may account for the negative findings in the experiment (No. 34b, Table III) with partly purified isomerase (13) and different concentrations of triose phosphate. Although the experiments involving the zymohexase equilibrium cannot be looked upon as final, they represent comparative measurements which favor the concept of an accumulation of an unstable addition product amounting, under our conditions, to 1 to 2 times the concentration of the glyceraldehyde monophosphate.

In Table IV data from three manometric experiments upon Baer and

Fischer's dimeric glyceraldehyde 1,3-diphosphate (7) are presented. The system consisted of the oxidizing enzyme, arsenate, bicarbonate, and either glyceraldehyde phosphate or the Baer-Fischer dimeric diphosphate. In the reaction 2 moles of H^+ (1 from glyceric acid phosphate and 1 from dihydrocozymase) develop per mole of cozymase reduced. Under our conditions with excess of glyceraldehyde phosphate the reduction was complete in 20 minutes. The data (Experiments 1 and 3) indicate that the dimeric glyceraldehyde diphosphate is inactive, and therefore cannot be the sought for intermediary. The diester, however, is hydrolyzable by heating in HCl to glyceraldehyde 3-phosphate (Experiment 2, Table IV). The synthetic diphosphate ester is also inactive in the presence of an active phosphorylase, prepared according to Green and Cori (14), which splits the aldehyde phosphoric group of glucose-1-phosphate (Experiment 3).[4] Hexose diphosphate does not react (Experiment 2), because the purified oxidizing enzyme is free of zymohexase.

Similar results were obtained spectrophotometrically in corresponding experiments with phosphate in place of arsenate. The Baer-Fischer diester was treated with 0.8 N HCl at 38°. A very slow decomposition occurred, which in 30 minutes amounted to 6 per cent of the total phosphate, equivalent to 12 per cent of the carbonyl phosphate split. When such a preparation with a total content of phosphate of 0.18 mg. per ml. was incubated with inorganic phosphate (pH 8.4), cozymase, and oxidizing enzyme, an extinction of 0.20 was obtained. The same value was yielded by a solution of 1×10^{-3} M *d*-glyceraldehyde phosphate, which corresponded to 7 per cent of the *d* component of the synthetic diester. Without pretreatment with acid, a value for extinction which corresponded to 2 per cent of the *d* component was obtained. This small reaction was accounted for by a slight spontaneous decomposition of the ester to glyceraldehyde monophosphate. After treating the diester at room temperature with alkali, which does not attack the Baer-Fischer ester but destroys the triose phosphate, it became completely inactive with reference to the reduction of cozymase.

SUMMARY

1. The equilibrium involved in the oxidation and phosphorylation of *d*-glyceraldehyde 3-phosphate, in the presence of cozymase and oxidizing enzyme, to *d*-glyceric acid 1,3-diphosphate has been investigated spectrophotometrically.

2. The reaction, under certain conditions which have been defined, does not obey simple mass law assumptions. Inorganic phosphate appears to have another function in the equilibrium besides that of simple phosphorylation or possible involvement in the formation of a precursor of glyceric acid diphosphate.

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[4] This experiment was suggested to us by Dr. H. O. L. Fischer.

3. Under specified conditions evidence has been obtained which is interpreted in favor of the existence of an intermediary, unstable addition product as a precursor of glyceric acid diphosphate.

4. The synthetic, dimeric glyceraldehyde diphosphate of Baer and Fischer (7) proved to be inactive. This diester therefore cannot serve as a precursor of glyceric acid diphosphate.

5. Dihydrocozymase can be titrated directly in neutral solution with iodine. The procedure is as quantitative and more simple and direct than Haas' earlier manometric determination with ferricyanide (9).

BIBLIOGRAPHY

1 Meyerhof, O., in Biological symposia, Lancaster, **5**, 141 (1941).
2. Lipmann, F., in Nord, F. F., and Werkman, C. H , Advances in enzymology and related subjects, New York, **1**, 99 (1941).
3. Warburg, O , and Christian, W , *Biochem Z* , **301**, 221 (1939), **303**, 40 (1939).
4. Negelein, E , and Bromel, H., *Biochem. Z.*, **301**, 135 (1939); **303**, 132 (1939).
5. Meyerhof, O , and Junowicz-Kocholaty, R., *J. Biol. Chem* , **149**, 71 (1943).
6. Meyerhof, O , and Junowicz-Kocholaty, R , *J Biol Chem* , **145**, 443 (1942).
7 Baer, E , and Fischer, H O. L., *J Biol Chem* , **150**, 213 (1943).
8. Fischer, H O L., and Baer, E , *Ber chem Ges* , **65 B**, 337 (1932).
9. Haas, E., *Biochem Z* , **291**, 79 (1937).
10 Drabkin, D L , *J Biol Chem* , **157**, 563 (1945)
11 Ohlmeyer, P , *Biochem. Z* , **297**, 66 (1938)
12. Warburg, O., and Christian, W., *Biochem Z* , **287**, 291 (1936)
13. Meyerhof, O., and Beck, L. V., *J Biol. Chem* , **156**, 109 (1944).
14. Green, A A , and Cori, G T , *J Biol. Chem* , **151**, 21 (1943)

A QUANTITATIVE METHOD FOR ETHANOLAMINE AND SERINE AS A BASIS FOR THE DETERMINATION OF PHOSPHATIDYL ETHANOLAMINE AND PHOSPHATIDYL SERINE IN TISSUES*

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In addition to ethanolamine which was formerly believed to be the sole nitrogenous component of cephalins, the hydroxyamino acid serine has been shown to be present in the cephalins of brain (1). Moreover, since the lipids of several other organs (2) and blood plasma (3) also contain amino acid N, it seems probable that serine-containing phospholipids are common constituents of animal tissues. Hence, there is a need for a practical method which would enable one to determine separately the amounts of ethanolamine- and serine-containing phospholipids in biological materials. The estimation of their nitrogenous constituents after hydrolysis[1] would probably represent the most convenient basis for such a method. Accordingly, the action of periodate on ethanolamine and serine has been reinvestigated with the intention of exploring its applicability to the determination of these substances in the hydrolysates of tissue lipids.

The reaction of periodate with serine was first shown by Nicolet and Shinn (5) to be quantitative, presumably according to the equation $HCH(OH)\cdot CH(NH_2)\cdot COOH + NaIO_4 = HCOH + CHO\cdot COOH + NH_3 + NaIO_3$. This equation is in agreement with the results of Van Slyke *et al.* (6) and also with those of the present study. As for the ethanolamine-periodate reaction, Van Slyke *et al.* (6) observed low recoveries of ammonia (determined by the aeration method), whereas Ramsay and Stewart (7) (employing Conway's diffusion method) obtained satisfactory yields. However, when the latter authors applied the procedure to hydrolyzed lipid extracts, they were able to recover only 65 to 75 per cent of the added ethanolamine.

In the present experiments, theoretical values for the periodate reduced and for the formaldehyde evolved in the reaction with ethanolamine were

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[1] Previous attempts to evaluate ethanolamine on the basis of its volatility at relatively low temperature have been reported by Blix (4)

obtained, according to the equation[2] $HCH(OH) \cdot CH_2(NH_2) + NaIO_4 = 2HCOH + NH_3 + NaIO_3$. Moreover, with the steam distillation procedure, quantitative recoveries of ammonia were observed after the action of periodate on both ethanolamine and serine. It was next found that these two substances can be separated by permutit, ethanolamine being quantitatively adsorbed, whereas serine is not adsorbed at all. Ethanolamine can be eluted subsequently with a concentrated solution of sodium chloride.

Since in the application of the method to tissue phospholipids these first have to be hydrolyzed, the action of certain hydrolytic agents on ethanolamine and serine was investigated. Hydrochloric acid in methanol did not cause any appreciable destruction of either compound, whereas considerable losses resulted from the treatment with an alcoholic solution of barium hydroxide. In the method proposed below, ethanolamine and serine are estimated from the ammonia liberated by periodate; consequently, the significance of the results is dependent upon the absence in the materials analyzed of other substances from which ammonia may be evolved during the procedure. Neither choline nor urea (a common contaminant of lipid extracts (8)) has been found to interfere. Among other known hydrolytic products of tissue lipids, some, such as glycerol and galactose, do react with periodate but obviously cannot yield ammonia. Because of the presence of adjacent amino and hydroxyl groups (9), sphingosine almost certainly yields ammonia by reacting with periodate. An attempt has been made to remove the amino alcohol from the hydrolysates by both ether extraction and filtration. However, it seems probable that this treatment may not have been completely successful in certain materials, such as brain lipids which contain large proportions of sphingomyelins and cerebrosides. Even in lipid extracts from other sources, the possibility remains that some unidentified constituents may still be present which could evolve ammonia by the action of periodate. Aside from these reservations on the significance of the results, the procedure appears to be reasonably accurate: known amounts of ethanolamine and serine, added to lipid extracts from tissues, have been recovered almost quantitatively.

Determination of Ethanolamine and Serine in Solutions of Pure Substances

On one fraction of the solution, the total amount of ethanolamine + serine is estimated. Another fraction is passed through a column of permutit and serine is determined in the filtrate. Ethanolamine may then be estimated either by difference or by a direct determination in the eluate.

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[2] Ramsay and Stewart (7) write the equation in the following form: $CH_2OH \cdot CH_2NH_2 + IO_4^- \rightarrow IO_3^- + HCHO + H_2O + NH_3$, suggesting that they could recover only 1 mole of formaldehyde for each reacting mole of ethanolamine.

The ammonia resulting from the reaction with periodate is distilled with steam into a solution of standard acid and the excess acid determined iodometrically.

Reagents—Permutit, according to Folin; sodium chloride, 30 per cent; alkaline borate solution (12.404 gm. of boric acid dissolved in 150 cc. of 1.0 N NaOH; 3.5 cc. of this buffer, when mixed with 2 cc. of 0.2 M periodic acid, yield a solution of pH 9.6, approximately); periodic acid ($HIO_4 + 2H_2O$),[3] 0.2 M; sulfuric (or hydrochloric) acid, 0.01 N (or 0.005 N); sodium thiosulfate, 0.005 N; soluble starch, 1 per cent. All reagents should be chemically pure. They should be free of ammonia and protected against its absorption from the air.

Apparatus—For the permutit adsorption, a piece of glass tubing 120 mm. long and 7 mm. external diameter, with one end slightly constricted and stoppered with a piece of cotton.[4] The tube is filled with 1.5 gm. of permutit and the unconstricted end connected by a piece of rubber tubing to the stem of a small funnel.

For both the reaction with periodate and the distillation of NH_3, the Pregl distillation apparatus, as modified by Parnas and Wagner (11).

Procedure—The solution (preferably containing 0.6 to 3 mg. of ethanolamine or 1 to 5 mg. of serine (or both) in a volume of about 5 cc.) is sent through the permutit column, which is then washed with a few cc. of distilled water. Ethanolamine may be eluted subsequently with 5 cc. of the sodium chloride solution, followed by three washings with 1 or 2 cc. of water each.

The determinations of ammonia are then carried out as follows: In the receiving flask of the Parnas-Wagner apparatus are measured accurately 5 or 10 cc. of 0.01 N sulfuric acid. In the digestion flask are placed, in the order given, (*a*) the solution containing serine or ethanolamine (or both), (*b*) 1 drop of paraffin oil, (*c*) 3.5 cc. of the alkaline borate solution, (*d*) 2 cc. of the periodic acid solution.[5] The stop-cock connecting the funnel to the digestion flask is closed immediately after each addition.[6]

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[3] G Frederick Smith Chemical Company, Columbus, Ohio.

[4] A similar adsorption apparatus has been used by Dubnoff and Borsook (10) for the removal of arginine, a stage preparatory to the determination of glycocyamine in biological materials

[5] In the aeration procedure employed by Van Slyke *et al.* (6) for the determination of hydroxyamino acids, an excess of glycine is added to the reaction mixture in order to make the ammonia yield quantitative. When the ammonia is separated by steam distillation, this addition becomes unnecessary.

[6] The steam distillation may be carried out under reduced pressure, as used by Parnas and Heller (12) in their determinations of NH_3 in blood. With such an arrangement, the liquid in the distilling flask can be maintained at a much lower temperature. However, identical results were obtained on hydrolysates of lipid extracts from tissues, with either this procedure or the simple steam distillation.

Steam is then passed through the liquid for 7 minutes. The receiving flask is lowered, so that the tip of the condenser remains above the level of the standard acid solution, and the distillation continued for 1 minute. The tip is then washed with a little water into the flask. 2 cc. each of potassium iodide and potassium iodate solutions are added. The flask is stoppered. After 5 minutes, the liberated iodine is titrated to a faint yellow color with 0.005 N thiosulfate by means of a micro burette graduated in divisions of 0.05 or 0.01 cc., the titration is completed after addition of 10 drops of the starch solution. With each series of analyses, a blank is run on all the reagents in the amounts used and the results of the titration corrected accordingly.[7]

Calculations—1 cc. of 0.005 N thiosulfate (corrected for the blank) = 0.303 mg. of ethanolamine = 0.525 mg. of serine.

Application of Procedure to Determination of Phosphatidyl Ethanolamine and Phosphatidyl Serine in Tissues

The lipids, extracted from the tissues, are hydrolyzed with methanolic HCl, which is later removed. The lipid hydrolysate is dissolved in water and the aqueous solution extracted with ethyl ether (or petroleum ether) and filtered. In the filtrate ethanolamine and serine are then determined.

Extraction and Hydrolysis of Lipids—Larger samples of tissue (10 to 15 gm.) are dehydrated with cold alcohol, then extracted with boiling alcohol in a continuous extraction apparatus. The alcohol is removed at about 45° under reduced pressure, the dry residue dissolved in chloroform, and the solution filtered through asbestos. On smaller samples (4 to 6 gm.), a simpler procedure, previously described (13), may be used. From a suitable aliquot of the chloroform solution,[8] the solvent is evaporated and the residue refluxed for 3 hours with 5 cc. of a 6 N solution of HCl in methanol. This is then removed at 60° under reduced pressure; the elimination of the HCl should be complete or nearly complete, since the adsorption of ethanolamine in strongly acid solution is not quantitative (Table II). Under the conditions described, no losses of either serine or ethanolamine were detectable.

Extraction and Filtration of Solutions of Lipid Hydrolysates—The dry residue is dissolved in 5 cc. of water and the solution extracted with three portions of either ethyl ether or petroleum ether (20, 15, and 10 cc.).[9] The extracts are discarded. The aqueous solution is freed from the last traces of the organic solvent with a stream of air, then filtered through an asbestos filter covered with a layer of sand. After washing the filter, the filtrate is brought to 12 cc. and divided into two fractions, one (4 cc.) for the determination of the sum of ethanolamine and serine, the other (8 cc.) for the determination of serine, after the adsorption of ethanolamine on permutit. Both fractions are neutralized to phenolphthalein and the reaction with periodate and distillation of ammonia carried out, as described.

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[7] The adsorption and titration of ammonia in boric acid solution have also been employed successfully 5 cc of saturated boric acid solution and 2 drops of 0 1 per cent solution of methyl red (or brom-cresol green) are placed in the receiving flask of the Parnas-Wagner apparatus After distillation of the ammonia, the solution is titrated with 0.01 N acid to the same color as that of the titrated blank. 1 cc. of 0.01 N acid = 0.606 mg. of ethanolamine = 1.05 mg. of serine

[8] In those materials in which larger proportions of sphingosine combinations are present, a fractionation of the chloroform extract is probably required (see some of the results reported in the following paper).

The determination of ethanolamine by difference is somewhat shorter. Moreover, the results obtained by this procedure can be checked by a direct determination in the eluate. The latter method must of course be employed if the amounts of material available are limited.

Blank Analyses—For the determinations of serine after permutit adsorption the blank analysis is carried out on the reagents used in the neutralization of the filtrate and in the evolution and distillation of ammonia. For the determination of the total ethanolamine + serine, and for that of ethanolamine in the eluate, the blank should also include the methanolic HCl employed for the hydrolysis of lipids, as this may contain appreciable amounts of ammonia.

Calculations—In the present status of our knowledge, it is probably more correct to report the results of the titrations directly as phospholipid equivalents. However, if one prefers to convert the data into approximate weights of phospholipids, the following relationships may be used. 1 cc. of 0.005 N thiosulfate (corrected for the blank analysis) = 3.75 mg. of phosphatidyl ethanolamine = 3.95 mg. of phosphatidyl serine.

EXPERIMENTAL

Reduction of Periodate by Ethanolamine and dl-Serine and Formation of Formaldehyde (Table I)—To pure solutions of ethanolamine,[10] *dl*-serine,[10] and choline hydrochloride,[10] an excess of periodic acid was added. The reaction mixture was made slightly alkaline to phenolphthalein and the volume adjusted to 20 cc.[11] After 20 minutes at room temperature, 1 drop of methyl red solution was added and the mixture brought with standard acid to an orange color. Then 10 cc. of a phosphate buffer (pH 6.0) and 2 cc. of 10 per cent potassium iodide were added, and the liberated iodine titrated with standard thiosulfate. Under these conditions, two thiosulfate equivalents titrate 1 mole of periodic acid, whereas no iodine is liberated from the iodic acid formed in the reduction of periodic acid. Formaldehyde was determined by precipitation with dimedon, according to Vorländer (14). After crystallization from alcohol, the melting point of the precipitate obtained from ethanolamine corresponded to that given for the dimedon derivative of formaldehyde (189° (14)). From Table I, it is clear that in the reaction with ethanolamine 1 mole of periodic acid is reduced and 2 moles of formaldehyde are formed. With serine or serine-ethanolamine mixtures the amounts of reduced periodate were definitely higher than expected (probably because of secondary reactions with glyoxylic acid formed from serine (6)). Urea and choline hydrochloride did not react with periodate.

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[9] It was found convenient to perform these extractions in centrifuge tubes of 50 cc. capacity, the ether layer being siphoned off after each extraction. In case of troublesome emulsions, the mixture may be centrifuged.

[10] Merck and Company. The solutions were standardized by the following methods. Ethanolamine in aqueous solution was directly titrated against standard acid with methyl red; *dl*-serine in 50 per cent dioxane was titrated against standard alkali with thymolphthalein; in choline hydrochloride solutions, N and Cl were determined.

[11] The results were not affected by variations in the volume of the mixtures (5 to 30 cc.), in the time of reaction (5 minutes to 2 hours) and in the initial pH (7 to 10). However, in acid solutions periodic acid appeared to be reduced at a definitely slower rate.

TABLE I

Reduction of Periodate and Formation of Formaldehyde from Ethanolamine and Serine

Most values are the average of duplicate analyses. When larger series of analyses have been made, the range of the values obtained in each series is given in parentheses.

Present			HIO_4 reduced to HIO_3		HCOH formed	
Ethanolamine	*dl*-Serine	Other substances		Recoveries		Recoveries
micromoles	*micromoles*	*micromoles*	*micromoles*	*per cent*	*micromoles*	*per cent*
400			395	99		
300			305	102		
200			200	100	394	98
			(195–205)	(97–102)	(378–405)	(94–101)
150			152	101	292	97
			(148–156)	(99–104)	(285–300)	(95–100)
100			100	100	200	100
			(98–102)	(98–102)	(190–206)	(95–103)
50			50	100	101	101
			(49–52)	(98–104)	(96–108)	(96–108)
25			24.5	98	51	102
			(24.3–25.1)	(97–100)	(50–55)	(100–110)
	300		335	112	290	97
300	200		550	110		
200	100	Choline HCl, 500	339	113	475	95
100	300	Urea, 500	465	116	470	94
50	50	Choline HCl, 500	112	112	144	96
		" " 500	0	0		
		Urea, 500	0	0		

Formation of Ammonia from Ethanolamine and Serine (Table II)—With the method used for the determination of ammonia theoretical yields were obtained from ethanolamine as well as from serine or from mixtures of both substances. From the results obtained in the filtrate after adsorption and in the eluate, it is apparent that the procedure described for their separation is satisfactory. Neither choline nor urea interferes. However, in the presence of comparatively large amounts of hydrochloric acid or sodium chloride the adsorption of ethanolamine on permutit becomes incomplete.

TABLE II

Formation of Ammonia from Ethanolamine and Serine (or Both) in Reaction with Periodate

Ethanolamine	*dl*-Serine	Other substances	NH_3 periodate			Recoveries*		
			Before adsorption	After adsorption	Eluate	Ethanolamine + serine	Ethanolamine	Serine
micromoles	*micromoles*		*micromoles*	*micromoles*	*micromoles*	*per cent*	*per cent*	*per cent*
100			99.3	0.5		99	99	
50			50.8	1.0	49.5	102	99†	
30			29.1	0 6		97	95	
20			19.5	0.2	19.0	98	95†	
	100		101.0			101		
	50		49.4	49.0		99		98
	30		29.0	29.2		97		97
	20		20.5	19.3		103		97
		Choline HCl, 100 micromoles	0					
		Urea, 100 micromoles	0.1					
50	50		101.5	48.3		102	106	97
50	50	Choline HCl, 100 micromoles	96.7	49.1	47.8	97	96†	98
50	30		81.5	29.5		102	104	98
50	15	Urea, 100 micromoles	67.7	14.3		104	107	95
50	15	" 100 "		14.5	48.5	97	97†	97
30	50	Choline HCl, 100 micromoles	79.5	49.0		99	102	98
15	50	" " 100 "	65.5	49.1	14.2	101	94†	99
50	30	N HCl, 2 cc	77.9	39.2		97	77	127
50		" " 2 "	49.5	12.0		99	75	
	50	" " 2 "	50.3	49.8		101		100
50		" NaCl, 2 cc.	49.2	14.5		98	69	

* Recoveries of ethanolamine are calculated from the differences between the results obtained on the solutions before and after adsorption, except for the values marked with † which are calculated on the basis of the determinations made on the eluate.

Recovery of Ethanolamine and Serine after Treatment with $Ba(OH)_2$ in Ethanol or with HCl in Methanol (Table III)—Small amounts of ethanolamine and serine were treated with $Ba(OH)_2$ in alcohol, according to the procedure used by Erickson *et al.* (15) for the hydrolysis of choline-containing phospholipids. This treatment resulted in considerable losses of ethanolamine, and, less so, of serine. In other experiments, the same amounts of ethanolamine and serine were refluxed for 3 hours with methanolic HCl and subsequently treated exactly as described for the application of the method to the hydrolysates of tissue phospholipids. Here satisfactory recoveries of both substances were observed.

TABLE III

Recoveries of Ethanolamine and Serine after Treatment with $Ba(OH)_2$ in Ethanol or HCl in Methanol

Ethanolamine	*dl*-Serine	$Ba(OH)_2$ in ethanol		HCl in methanol	
		NH_3 periodate	Recoveries	NH_3 periodate	Recoveries
micromoles	*micromoles*	*micromoles*	*per cent*	*micromoles*	*per cent*
50		20	40	51	102
	50	46	92	49	98
50	50	59	59	96	96
50	25	47	63	72	96
25	50	69	92	74	99

TABLE IV

Removal of Interfering Substances by Extraction with Ether and Filtration of Lipid Hydrolysates

Rat tissue	NH_3 periodate		Non-choline-containing phospholipids (by difference)
	Before treatmen	After treatment	
	micromoles	*micromoles*	*micromoles*
Liver (Extract I)	16.4	14.3	13.8
" (" II)	20.8	15.9*	17.4
Same + 5 micromoles each of ethanolamine and serine	30.9	26.4*	
Muscle (Extract I)	7.1	6.6*	6.4
" (" II)	6.7	6.3	7.0
Brain (" I)	59.0	52.6	41.3
" (" II)	61.2	52.3	41.7

* Extraction with petroleum ether.

Removal of Interfering Substances from Lipid Hydrolysates (Table IV)—The NH_3 liberated by periodate has been determined on two fractions of the same lipid hydrolysate, before and after extraction with ether (or petroleum ether) and filtration through asbestos filters. The amounts of non-choline phospholipids present in the tissues have also been estimated from the difference between the values for total and choline-containing phospholipids (calculated from the results of phosphorus and choline analyses, respectively).

It is apparent that the suggested treatment removes not negligible amounts of substances (other than ethanolamine and serine) which evolve ammonia by reacting with periodate. After removal of these substances, the values obtained by the present procedure on liver and muscle extracts corresponded closely to those calculated for the non-choline phospholipids, whereas they were still definitely higher in the hydrolysates of brain lipids.

TABLE V

Recoveries of Ethanolamine and Serine Added to Lipids Extracted from Rat Livers

Lipid extract No	Ethanolamine		Serine		NH_3 periodate			Total recoveries		
	In extract	Added	In extract	Added	Before adsorption	After adsorption	Eluate	Ethanolamine + serine	Ethanolamine	Serine
	micromoles	*micromoles*	*micromoles*	*micromoles*	*micromoles*	*micromoles*	*micromoles*	*per cent*	*per cent*	*per cent*
III	19.0	35	5.0	15		21	53.0	100	98	105
	19.0	30	5.0	15	67.6			98		
	19.0	15*	5.0	35*		40.5	33.0	99	97	101
	12.7	20	3.3	9.5	46.1	12.6		101	102	98
	12.7		3.3	9.5		12.4	12.6	98	99	97
	12.7		3.3	14.3*		17.5	12.2	98	96	99
	12.7	10	3.3	19.0	44.0	21.0		98	101	94
	12.7		3.3	28.5		30.5			96	
IV	15.8	20*	4.2	20.0*	59.0	24.5		98	99	101
	15.8	6	4.2		27.0		22.5	104	103	107
	7.9	25*	2.1		35.0			100		
	7.9		2.1	25.0	35.0	28.0		100	89	103
	7.9	20	2.1	20.0		24.5	26.0	101	93	111

* Substances added to the extracts before hydrolysis.

Similar findings on lung and kidney lipids will be reported and discussed in the following paper.

Recoveries of Ethanolamine and Serine Added to Lipid Extracts of Tissues (*Table V*)—In these experiments, the chloroform solutions of the lipids, extracted as described from the pooled livers of two groups of rats, Extracts III and IV, were used. Variable amounts of ethanolamine and serine were added to aliquots of the solutions, usually after hydrolysis with HCl in methanol. The total recoveries on the samples before adsorption were practically complete. As could be expected, the results obtained after adsorption and elution are somewhat less accurate but even in the determination of individual substances, the error usually did not exceed 5 per cent of the calculated values.

The writer is indebted to Dr. W. H. Fishman for helpful advice and criticism.

SUMMARY

Ethanolamine and serine may be determined quantitatively in a mixture of the pure substances. The procedure involves the reaction with alkaline periodate before and after ethanolamine is removed by adsorption on permutit. The NH_3 evolved is separated by steam distillation and determined.

The conditions for applying this method to the estimation of ethanolamine- and serine-containing phospholipids in tissues are outlined.

Addendum—Since this manuscript went to press, an abstract has appeared of a paper by Jones (16). His data for the ethanolamine-periodate reaction are in complete agreement with those presented here.

BIBLIOGRAPHY

1. Folch, J., and Schneider, H. A., *J. Biol. Chem.*, **137**, 51 (1941) Folch, J., *J. Biol. Chem*, **139**, 973 (1941), **146**, 35 (1942) Chargaff, E., and Ziff, M., *J. Biol. Chem.*, **140**, 927 (1941). Schuwirth, K., *Z. physiol Chem*, **277**, 87 (1942).
2. Chargaff, E, Ziff, M., and Rittenberg, D, *J. Biol Chem*, **138**, 439 (1941); **144**, 343 (1942)
3. Folch, J., Schneider, H. A., and Van Slyke, D. D., *Proc Am. Soc Biol. Chem.*, *J. Biol. Chem*, **133**, p. xxxiii (1940).
4. Blix, G., *Biochem. Z.*, **305**, 136 (1940).
5. Nicolet, B. H., and Shinn, L. A., *J. Am. Chem Soc*, **61**, 1615 (1939); *J Biol. Chem.*, **139**, 687 (1941)
6. Van Slyke, D. D., Hiller, A., and MacFayden, D. A, *J. Biol Chem.*, **141**, 681 (1941).
7. Ramsay, W. N. M., and Stewart, C P, *Biochem J*, **35**, 39 (1941).
8. Christensen, H. N., *J. Biol. Chem.*, **129**, 531 (1939). Folch, J., and Van Slyke, D. D., *J. Biol. Chem.*, **129**, 539 (1939).
9. Carter, H. E., Glick, F. J., Norris, W. P, and Phillips, G E, *J. Biol. Chem*, **142**, 449 (1942).
10. Dubnoff, J. W., and Borsook, H, *J Biol. Chem.*, **138**, 381 (1941).
11. Parnas, J. K., and Wagner, R., *Biochem. Z.*, **125**, 253 (1921)
12. Parnas, J. K, and Heller, J., *Biochem. Z.*, **152**, 1 (1924).
13. Artom, C., and Fishman, W. H., *J. Biol. Chem*, **148**, 405 (1943).
14. Vorländer, D., *Z. anal. Chem.*, **77**, 241 (1929).
15. Erickson, B. N., Avrin, I., Teague, D. M., and Williams, H. H., *J. Biol. Chem.*, **135**, 671 (1940).
16. Jones, J. H., *J. Assn. Official Agr. Chem.*, **27**, 462 (1944); *Chem. Abstr.*, **38**, 6276 (1944).

SOME DATA ON THE DISTRIBUTION OF INDIVIDUAL PHOSPHOLIPIDS IN RAT TISSUES AND IN HUMAN PLASMA*

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A quantitative method for ethanolamine and serine has been described and its possible application to the determination of phosphatidyl ethanolamine and phosphatidyl serine in tissues has been pointed out (1). By employing this procedure, data on the amounts of these phospholipid fractions in some rat tissues and in human plasma have been obtained. Since the total and choline-containing phospholipids have also been evaluated, a rather detailed picture of the distribution of individual phospholipids in these materials may be outlined.

EXPERIMENTAL

Three groups of male albino rats, raised on a stock diet (Rockland Farms, complete) to about 150 gm., were killed by decapitation and the determinations made on pooled samples of their tissues. Human plasma (120 cc.) was separated by centrifugation of citrated blood, collected from various individuals. The procedures used for the extraction and purification of lipids from the tissues of rats (1, 2) and from human plasma ((3), method (*c*)) have been described. On aliquots of the chloroform solutions of lipids thus obtained, after wet combustion or hydrolysis, phosphorus (4), choline (2), ethanolamine and serine (1) were determined.

In one analysis of kidney and of brain, a portion of the chloroform solutions, after removal of the solvent, has been further treated with ethyl ether. The ether-insoluble material which settled out immediately (kidney) or after standing for several weeks (brain) was separated by centrifugation, and the determinations repeated on the clear ether solutions.

In Table I the results are expressed as micromoles of phospholipids for 1 gm. of moist tissue or 100 cc. of plasma. For this purpose, the weights of the total phospholipids have been first calculated from the phosphorus figures, by use of the empirical factor 22.7 (previously determined in rat liver and muscle (2)). By dividing by 0.775 (25 × 0.031), the weights in mg. were then converted into micromoles of total phospholipids. The equivalent values for the individual phospholipid fractions have been

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obtained directly from the titration data of the choline, ethanolamine, and serine determinations. Only the non-choline-containing phospholipids were calculated from the difference between the total and the choline-containing phospholipids.

TABLE I

Distribution of Individual Phospholipids in Some Tissues of Rats and in Human Plasma

Values in micromoles for 1 gm. of moist tissue or 100 cc. of plasma.

Material	Rat group	Total phospholipids	Choline-containing phospholipids	Non-choline-containing phospholipids				
				Total (a − b)	Ethanolamine-containing	Serine-containing	Ethanolamine- + serine-containing (d + e)	Difference from calculated values (f−c)
		(a)	(b)	(c)	(d)	(e)	(f)	(g)
Liver	A	39.8	22.4	17.4	10.9	4.5	15.4	−2.0
	B	38.2	24.4	13.8	10.4	3.9	14.3	+0.5
	C	39.2	23.5	15.7	12.6	2.9	15.5	−0.2
Skeletal muscle	A	12.1	5.7	6.4	4.6	2.0	6.6	+0.2
	B	12.7	5.7	7.0	5.6	0.7	6.3	−0.7
	C	12.9	6.6	6.3	4.4	1.5	5.9	−0.4
Heart	A	20.8	5.4	15.4	10.1	1.5	11.6	−3.8
	B	20.3	6.4	13.9	10.4	2.3	12.7	−1.2
Testis	A	16.1	8.4	7.7	5.5	2.1	7.6	−0.1
Spleen	"	17.2	7.8	9.4	6.1	2.9	9.0	−0.4
	B	15.3	4.7	10.6	8.1	3.6	11.7	+1.1
Lung	A	23.2	14.2	9.0	8.9	1.8	10.7	+1.7
	B	21.7	12.9	8.8	12.2	2.6	14.8	+6.0
Kidney	A	30.5	16.2	14.3	13.4	8.7	22.1	+7.8
	B	34.9	18.6	16.3	16.9	7.4	24.3	+8.0
	"*	29.8	12.3	17.5	16.4	2.1	18.5	+1.0
Brain	A	65.2	23.9	41.3	35.4	17.2	52.6	+11.3
	B	64.9	23.2	41.7	31.5	20.8	52.3	+10.6
	"*	57.7	22.5	35.2	23.2	12.4	35.6	+0.4
Human plasma†		197	131	66	42	13	55	−11

* Ether-soluble fraction of the lipid extract.

† On a fraction of the chloroform solution, the sphingomyelins were precipitated with Reinecke acid and the P determined in the precipitate (2). From the combined data of all determinations, it appears that, in the sample of plasma analyzed, lecithins (choline-containing phospholipids less sphingomyelins) represented 55, phosphatidyl ethanolamine 21, phosphatidyl serine 7, and sphingomyelins 12 per cent of the total phospholipids.

DISCUSSION

In most of the materials analyzed, the sum of the ethanolamine- and serine-containing phospholipids, determined directly, approximates the value for the non-choline-containing phospholipids, estimated by difference.

Here the discrepancies are of such a small extent that they may be explained by the limits of error of the methods and by the use of an arbitrary factor for the calculation of total phospholipids. When the differences are significant, as in the heart, they may be suggestive of the presence of unknown phospholipids (5) or of other compounds, such as phosphatidic acids and "cardiolipin" (6), which contain phosphorus but no nitrogen.

On the other hand, in the lung, kidney, and brain, the sum of the ethanolamine- plus serine-containing phospholipids is considerably greater than the amount of the non-choline-containing phospholipids. As pointed out previously (1), the higher value could be ascribed to an incomplete removal of sphingosine (either free or combined in products of partial hydrolysis, whose solubility characteristics may be different from those of the free base); such a cause of error would, of course, become more important in materials in which larger proportions of sphingomyelins[1] (or cerebrosides, or both) are present.[2] At any rate, it is apparent that in the ether-soluble fractions of the brain and kidney lipids, the sum of phosphatidyl ethanolamine and phosphatidyl serine corresponds closely to the value for the non-choline-containing phospholipids.[3]

Data on the amounts of total and choline-containing phospholipids in tissues of various animals, including rats, and in human plasma have been reported in a number of papers from this (2, 3) and other laboratories. Most of these previous data agree fairly well with the values reported here.[4] On the other hand the only results of the previous literature with which the present values for ethanolamine- and serine-containing phospholipids can be compared are those published by Chargaff *et al.* (5) on phospholipid mixtures isolated from pig and beef tissues. Even this comparison is somewhat arbitrary, as in their analyses the preliminary purification of the materials may have caused losses to a different degree in the various phospholipid fractions. If this point, as well as the differences in the animal species and in the procedures employed are taken into consideration, the general agreement between their values and those of the present investigation may be regarded as satisfactory (Table II).

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[1] In the brain, lungs, and kidneys the sphingomyelins represent between 19 and 33 per cent (cat tissues (7)) or between 15 and 25 (beef tissues (8)) per cent of the total phospholipids, whereas in the liver, heart, and skeletal muscles they constitute only 7 to 9.5 per cent (cat) or 4 to 6 (beef) per cent of the total phospholipids.

[2] There are of course other possible explanations such as the existence of diamino phospholipids with a constitution other than that of sphingomyelins, or the presence in the lipid hydrolysates of undetermined nitrogenous components which also might liberate ammonia by reacting with periodate. These possibilities do not appear unreasonable when the very recent advances in this field are considered (9-11) In this connection, it may also be mentioned that in the determinations by Chargaff *et al.* (5) 50 per cent of the non-amino N in the phospholipids of pig heart and beef brain could not be identified as choline N. Moreover, 36 per cent of the amino N in the phospholipids of pig liver was present in compounds other than ethanolamine or amino acid

[3] From the data reported in Table I, it may be calculated that in the ether-insoluble fraction, separated from the crude kidney extracts, the molecular ratios of P to choline to NH_3 periodate were 1:1 24:1.14, which approximate those expected for sphingomyelins. However, in the ether-insoluble portion of brain lipids the corresponding ratios were 1:0 1:2.32 A discussion of the significance of these findings would at present be merely speculative.

[4] A comprehensive study on the distribution of lipids in the tissues of various animals has been published recently by Kaucher *et al.* (8). For additional information on the subject, the reader is referred to the articles on fat metabolism in the recent volumes of the "Annual review of biochemistry."

At any rate, since the specificity of the method employed in the present analyses is only relative, the data reported here merely represent the maximum possible values for the amounts of ethanolamine- and serine-containing phospholipids in plasma and tissues. Accordingly, it may be stated that, while the latter group of phospholipids is probably present in all tissues, it represents only a minor fraction of the total and also of the non-choline-containing phospholipids.

TABLE II

Comparison between Previous (5) and Present Data on Amounts of Ethanolamine- and Serine-Containing Phospholipids in Some Tissues

	Method	Tissue			
		Liver	Heart	Lung	Brain
Ethanolamine N *(5)	Isotope dilution	27.2	38.6		44.4
Ethanolamine- containing phospholipids†	NH_3 periodate	27 3-32.2	48.6, 51.2		40.2‡
Lipid amino acid N *(5)	Ninhydrin	6.7	7.0	15.7	21.6
Serine-containing phospholipids†	NH_3 periodate	7 4-11.3	7.2, 11.3	7.8, 12.0	21.5‡

* Per cent of total lipid N (beef or pig tissues)

† Per cent of total phospholipids (rat tissues).

‡ Ether-soluble fraction.

SUMMARY

Total, choline-, ethanolamine-, and serine-containing phospholipids have been determined in several tissues of rats and in human plasma.

Serine-containing phospholipids have been found in all materials analyzed. However, they represent only a minor fraction of the total and also of the non-choline-containing phospholipids.

BIBLIOGRAPHY

1. Artom, C., *J. Biol. Chem*, **157,** 585 (1945).
2. Artom, C., and Fishman, W. H, *J. Biol Chem*, **148,** 405 (1943)
3. Artom, C, *J. Biol Chem*, **139,** 65 (1941)
4. Tisdall, F. F, *J Biol Chem*, **50,** 329 (1922).
5. Chargaff, E., Ziff, M., and Rittenberg, D., *J. Biol. Chem*, **144,** 343 (1942).
6. Pangborn, M. C., *J. Biol. Chem.*, **143,** 247 (1942).
7. Hunter, F. E., *J. Biol. Chem.*, **144,** 439 (1942).
8. Kaucher, M., Galbraith, H., Button, V., and Williams, H. H., *Arch. Biochem.*, **3,** 302 (1943)
9. Folch, J, and Schneider, H. A., *J. Biol. Chem.*, **137,** 51 (1941).
10. Folch, J, and Woolley, D. W., *J. Biol Chem.*, **142,** 963 (1942). Folch, J., *J. Biol. Chem.*, **146,** 35 (1942).
11. Klenk, E., *Z. physiol. Chem.*, **273,** 73 (1942). Klenk, E., and Rennkamp, F., *Z. physiol. Chem*, **273,** 253 (1942).

STEROID EXCRETION IN A CASE OF ADRENOCORTICAL CARCINOMA

II. AN EXAMINATION OF THE NON-KETONIC FRACTION PRECIPITABLE WITH DIGITONIN*

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In the course of an investigation of the steroids excreted by a boy with adrenocortical carcinoma (1) a triol of the androstene series was isolated which carried its hydroxyl groups in the same positions as estriol. In view of this structural similarity the question was raised whether the two compounds are formed by analogous reactions in the body. While this hypothesis has not yet been tested experimentally, it seemed of interest to investigate whether further similarities exist between the excretion products of the androstene and estratriene series. The latter of these in the human comprises estriol, estrone, and α-estradiol. While the 17-ketone of the androstene series, dehydroisoandrosterone, has long been known as a constituent of human urine, Δ^5-androstenediol-3(β),17(α), the analogue to α-estradiol, has not yet been isolated from natural sources. We have, therefore, searched for this compound by extending the analysis of the tumor urine to the non-ketonic fraction precipitable with digitonin.

This fraction was obtained as a complex mixture of crystalline material which could not be separated readily by fractional crystallization. It was therefore acetylated and subjected to chromatographic analysis. In this manner six different crystalline compounds were isolated. Two of these were identified as the diacetates of Δ^5-androstenediol-3(β),17(α) and of Δ^5-pregnenediol-3(β),20(α). A third substance is in all probability a Δ^5-pregnenediol-3(β),20(α) monoacetate, while the structure of the remaining three compounds has not yet been determined.

The compound present in largest amounts melted at 147°. Analysis of this product and its parent substance indicated that the isolated material was the diacetate of a diol, $C_{21}H_{34}O_2$. The only dihydroxysteroid with this formula which has previously been encountered in urinary extracts is Δ^5-pregnenediol-3(β),20(α). It was found in the urine of pregnant mares (2) by Marker who also obtained it in the degradation of diosgenin (3). While there was good agreement in the melting points of the diacetates

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(4), our diol possessed a melting point 9° higher than the highest value recorded for pregnenediol. In spite of this discrepancy our product was unequivocally identified as Δ^5-pregnenediol-3(β),20(α). Like Marker's compound our diol yielded upon oxidation progesterone and upon reduction allopregnanediol-3(β),20(α).[1] The identities of the reaction products were established by mixed melting point determinations with authentic specimens. The required allopregnanediol-3(β),20(α) and its diacetate were prepared from allopregnanediol-3(α),20(α) by epimerization with sodium (5). These reactions establish the nature of the carbon skeleton of the isolated diol and the positions and configurations of the hydroxyl groups and demonstrate the presence of a double bond between carbon atoms 5 and 6, or conceivably between 4 and 5. As the diol is stable towards acid and does not give the Rosenheim reaction, an allylic arrangement of ethylene and hydroxyl groups as is present in Δ^4-pregnenediol can be excluded (7).

The diacetate of pregnenediol was accompanied by small amounts of a higher melting compound which likewise yielded Δ^5-pregnenediol-3(β),20(α) upon hydrolysis. The analytical values agreed quite well with those demanded by the formula $C_{23}H_{36}O_3$. The substance is therefore regarded as a monoacetate of pregnenediol.

The second compound present in large amounts was identified as the diacetate of Δ^5-androstenediol-3(β),17(α). This structural assignment is based on the analyses, melting points, and mixed melting points of the isolated material and of its parent compound, and is confirmed by the conversion of the diol to Δ^4-androstenedione-3,17. The reference specimens were prepared from dehydroisoandrosterone (8–10)

The remaining three compounds which have not been identified are provisionally designated as Compound Ba, and as the acetates of Compounds A and C, respectively Compound Ba has been obtained in very small amounts and has been characterized only by its melting point (232.5–234.5°). Compound A gave analytical figures in close agreement with the formula $C_{21}H_{34}O_3$. The 3 oxygen atoms are present as hydroxyl groups in reactive positions as is indicated by the formation of a triacetate, $C_{27}H_{40}O_6$. The analysis of the acetyl derivative of Compound C suggests that it is a diacetate of a compound $C_{20}H_{32}O_3 \pm CH_2$. Additional data are required to clarify the composition of this product, but must await the accumulation of larger amounts of material.

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[1] Throughout this paper a hydroxyl group at C-20 is designated as "α" if its configuration is the same as in natural pregnanediol (pregnanediol-3(α),20(α)) (5). Pregnanediol and allopregnanediol-3(β),20(α) possess the same spatial arrangement at C-20, since Δ^5-pregnenediol can be converted by reactions not involving this asymmetric center to allopregnanediol-3(β),20(α) (2, 3) and to a Δ^4-pregnenol-20-one-3 (4) identical with the Δ^4-pregnenol-20(α)-one-3 prepared from pregnanediol-3(α),20(α) (6).

The isolation of these six substances accounts for 37 per cent of the acetylated, non-ketonic compounds which precipitated with digitonin. The remainder of the material is distributed rather evenly over the various eluates and the presence of additional compounds in large amounts, therefore, is not to be expected. We have failed so far to isolate cholesterol, the only constituent of this fraction that hitherto has been obtained from normal human urine. This was to be anticipated, however, since the volume of urine examined was insufficient for this purpose unless the concentration of cholesterol greatly exceeded that reported for the majority of cases of carcinoma (11).

Neither pregnenediol nor androstenediol has been found previously in human urine. While their absence from normal urine cannot be taken for granted, it seems out of the question that these compounds could have escaped detection if their concentrations in normal urine were comparable to those encountered by us. The yield of pregnenediol amounted to 35 mg. per liter, which exceeds that of the total non-ketonic fraction of the urine of normal men as reported by Callow (12) (22.4 mg. per liter). 7.7 mg. of androstenediol were isolated from 1 liter of urine, which is equivalent to about 15 I.U in the capon assay (13). The androgenic potency of a "low ketone" fraction of the urine of normal men has been found to be less than 2 I.U. per liter (14). Corresponding values for normal children appear not to be known but may be presumed to be even lower. One can conclude therefore that the two diols were derived largely if not entirely from adrenal tissue. Previous studies of the ketones excreted by patients with adrenocortical tumors have revealed a disproportionate increase in the output of dehydroisoandrosterone (15, 16). The isolation of considerable amounts of Δ^5-androstenetriol-3(β),16,17 (1), Δ^5-androstenediol-3(β),17(α), and Δ^5-pregnenediol-3(β),20(α) can be interpreted as additional examples of a trend towards the production of Δ^5-unsaturated β-3-hydroxysteroids (Δ^5-stenols) in cases of this type. This phenomenon is in striking contrast to the absence of Δ^5-stenols (other than cholesterol) from adrenal extracts. While the investigations on the steroid content of the adrenal have been carried out with cattle glands, the species difference presumably does not account for this dissimilarity in chemical composition. At least dehydroisoandrosterone has been found in the urine of bulls (17), cows (17),[2] and steers (18) just as it has been found in the urine of normal men (19) and women (20) and gonadectomized subjects of either sex (21, 22). Two other explanations can be adduced: (1) Steroids secreted by the adrenal are converted into Δ^5-stenols at some other site. (2) Δ^5-Stenols are formed but not stored in the adrenal cortex and are either secreted or promptly converted into other compounds such as β-3-hydroxysteroids of the *allo* type and Δ^4-unsaturated 3-ketosteroids. It would appear that a disproportionate increase in Δ^5-stenols in cases of adrenal neoplasm is explained more readily by the second hypothesis. This metabolic disturbance can then be regarded as the effect of an overproduction of Δ^5-stenols, which is not fully matched by their conversion into other compounds.

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[2] The presence of dehydroisoandrosterone in the urine of (pregnant) cows is indicated but not definitely established (17).

On this basis Δ^5-pregnenol-3(β)-one-20 is the most likely immediate precursor of pregnenediol to be suggested at this time. While pregnenolone has not been found in adrenal extracts, two substances which could be regarded as its metabolites, progesterone and allopregnanol-3(β)-one-20, have been isolated from this source (23). The ability of the body to reduce 20-ketosteroids to 20(α)-hydroxysteroids, which is postulated in the reduction of pregnenolone to pregnenediol, is exemplified by the metabolic conversion of progesterone to pregnanediol-3(α),20(α) (24). A similar reaction may account for the presence of androstenediol in this urine, since dehydroisoandrosterone is regarded as its likely precursor. So far experimental evidence has been adduced in the C_{19} series only for the conversion of 17(α)-hydroxysteroids into 17-ketosteroids (12, 25) but not for the reverse reaction. On the other hand the adrenal cortex in contrast to the testis appears to be lacking in 17(α)-hydroxysteroids. Adrenal extracts, however, have yielded 17-ketosteroids and 17(β)-hydroxy-20-ketosteroids (26) which have frequently been suggested as their precursors. It seems likely therefore that the situation is similar to that prevailing in the estrogen series in which in the human α-estradiol and estrone are mutually interconvertible (27, 28).[3]

It is now possible to supplement the report (1) on the isolation of Δ^5-androstenetriol-3(β),16,17 with the results of a bioassay of its triacetate. This derivative was used in preference to the parent compound on account of its greater solubility in suitable solvents. The assay for androgenic activity, which was carried out by Dr. R. I. Dorfman according to the procedure of Frank, Klempner, Hollander, and Kriss (30), revealed no growth of the chick comb at a dose level of 700 γ of Δ^5-androstenetriol-3(β),16,17 triacetate per animal. Simultaneous assay of androsterone showed a significant response upon application of 10 γ per animal.

[3] Since dehydroisoandrosterone can be reduced to Δ^5-androstenediol-3(β),17(α) by *Bacillus putrificus* (29), the possibility should be considered that the diol might have been formed in the voided urine by bacterial action. This contingency, however, is regarded as very unlikely, since the urine was collected under highly favorable conditions (1) which permitted preservation of 99 per cent of the neutral ether-soluble fraction in conjugated form (1).

EXPERIMENTAL[4]

Fractionation of Extracts—The neutral ether-soluble fraction of 4.2 liters of urine which had been freed of compounds insoluble in benzene (androstenetriol) served as starting material. This fraction (1) (which included the benzene extract of the mother liquors of androstenetriol) (11.75 gm.) was separated into ketonic and non-ketonic material by means of 16.4 gm. of betaine hydrazide according to Girard and Sandulesco (31).[5] The residue from the ether phase (2.4 liters) weighed 2.84 gm. (non-ketonic fraction). The ketonic fraction yielded 6.1 gm. of neutral products besides 0.52 gm. of material soluble in sodium hydroxide and 0.32 gm. of an alcohol-soluble precipitate that had formed at the water-ether interphase upon hydrolysis of the hydrazones.

The non-ketonic fraction was dissolved in 95 cc. of 80 per cent alcohol and treated with a hot solution of 2.4 gm. of digitonin in 105 cc. of 80 per cent ethanol. An unusually voluminous precipitate formed. After 48 hours it was separated by filtration and washed with 80 per cent alcohol and repeatedly with ether. (Small aliquots of the filtrate yielded precipitates upon the addition of cholesterol as well as of digitonin. This fraction therefore still contains small amounts of material forming moderately soluble digitonides.) The precipitate was freed of digitonin in the usual manner (32). The resulting product crystallized upon the addition of ethanol and was dried at room temperature (non-ketonic fraction precipitable with digitonin, 599 mg.). It was dissolved in 20 cc. of pyridine and 10 cc. of acetic anhydride and kept at room temperature for 16 hours. The reaction mixture was taken to dryness under reduced pressure and dissolved in ether. The solution was washed with dilute hydrochloric acid, sodium carbonate solution, and water and yielded upon evaporation 684 mg. of a yellow oil. This was dissolved in a mixture of 8 cc. of petroleum ether and 2 cc. of benzene and passed through a column (100 × 13 mm.) of alumina (according to Brockmann, manufactured by Merck and Company, Inc., Rahway, New Jersey). The adsorbed material was subjected to fractional elution as indicated in Table I.

Purification of Fraction 1—Fraction 1 was not readily purified by recrystallization. It was therefore dissolved in 8 cc. of a mixture of petroleum ether and benzene (2:5) and adsorbed on a column (11 × 77 mm.) of alumina. The chromatogram was developed with 10, 30, 30, 30, 30, and 100 cc. of a mixture of petroleum ether and benzene (4:1), with 50, 75, 100, and 100 cc. of a 1:1 mixture of these solvents, with 30, 40, 100, 100, and 200 cc. of benzene, with benzene containing acetone (50 cc. (2 per cent acetone), 50 cc. (5 per cent), 100 cc. (10 per cent), and 100 cc. (20 per cent)), and finally with 100 cc. of acetone. These solvents eluted 1.9, 2.3, 1.3, 0.6, 0.3, 1.5, 4.7, 6.2, 1.2, 0.4, 1.5, 3.0, 10.2, 4.9, 4.2, 0.6, 1.5, 3.3, 1.9, and 2.1 mg. of material respectively (Fractions 1-a to 1-t).

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[4] All melting points reported are corrected.

[5] The separation was carried out at a pH of 6.5, which requires the addition of larger amounts of alkali (sodium hydroxide) than the 0.9 neutralization suggested by Girard.

TABLE I

Chromatographic Analysis of Acetylated Non-Ketonic Material Precipitable with Digitonin

Fraction No.	Eluant		Weight of eluate	Compounds isolated
	Volume	Composition		
	cc		*mg.*	
1	25	Petroleum ether + benzene, 4:1	62.9	Δ^5-Pregnenediol-3(β),20(α) diacetate Δ^5-Pregnenediol-3(β),20(α) monoacetate
2	120	" " + " 4:1	172.4	Δ^5-Pregnenediol-3(β),20(α) diacetate
3	100	" " + " 4:1	30.5	" "
4	150	" " + " 4:1	18.7	" "
5	175	" " + " 4:1	13.4	Partly crystalline
6	125	" " + " 1:1	37.1	Δ^5-Androstenediol-3(β), 17(α) diacetate
7	125	" " + " 1:1	22.4	" "
8	150	" " + " 1:1	16.0	" "
9	175	" " + " 1:1	12.2	" "
10	110	" " + " 1:1	5.7	Partly crystalline
11	100	Benzene	27.1	Acetate of Compound A
12	125	"	15.2	" " " "
13	150	"	13.7	Oil
14	150	"	15.1	Compound Ba
15	100	" + acetone, 4:1	134.7	Acetate of Compound C
16	100	" + " 4:1	6.1	Oil
17	250	" + " 4:1	5.5	"
18	100	Acetone	7.1	Partly crystalline
19	185	"	3.4	" "
20	175	Methanol	31.7	

Fractions 1-a, 1-b, and 1-c while crystalline yielded no homogeneous product. Fractions 1-g, 1-h, and 1-i were combined and recrystallized from methanol. 4.1 mg. of thin platelets melting at 145–146.5° were obtained. The mother liquors yielded 2.9 mg. of crystals melting at 143–145°. Admixture with Δ^5-pregnenediol-3(β),20(α) diacetate did not depress the melting point.

Fractions 1-m and 1-n were recrystallized from methanol, from petroleum ether, and from dilute methanol. 4.0 mg. of hexagonal plates were obtained which melted at 164–168°. The melting point was depressed upon admixture with either allopregnanediol-3(β),20(α) diacetate or Δ^5-androstenediol-3(β),17(α) diacetate.

Analysis—Sample dried at 80° *in vacuo*
$C_{23}H_{36}O_3$. Calculated, C 76.62, H 10.07; found, C 76.24, H 10.10

The mother liquors of these two fractions were combined with Fractions 1-l and 1-o and yielded an additional 4.0 mg. of less pure material melting at 156–166°, which was hydrolyzed in the manner described below. The reaction product which was recrystallized from acetone and extracted with a small volume of ether showed no depression of its melting point (182.5–185°) upon admixture with Δ^5-pregnenediol-3(β),20(α). The yield was 2.7 mg.

Isolation of Δ^5-Pregnenediol-3(β),20(α) Diacetate—Fraction 2 was repeatedly recrystallized from methanol. 109 mg. of hexagonal plates melting at 146–147° were obtained. An allotropic modification melting at 137° frequently formed in the melt on resolidification. Occasionally the diacetate crystallized from methanol in needles.

Analysis—Sample dried at 80° *in vacuo*
$C_{25}H_{38}O_4$. Calculated, C 74.59, H 9.52; found, C 74.67, H 9.55

Fractions 3 and 4 were purified in the same manner and yielded 12.5 mg. (m.p. 145–147°) and 9.9 mg. (m.p. 144–146°) respectively of the same compound. An additional 38.1 mg. of these crystals (m.p. 142–145.5°) were obtained from the combined mother liquors. The identity of these products was confirmed by mixed melting point determinations. Pregnenediol diacetate has been reported to melt at 144–146° (4).

Hydrolysis of Δ^5-Pregnenediol-3(β),20(α) Diacetate—A solution of 18.1 mg. of pregnenediol diacetate and 32 mg. of sodium hydroxide in 5 cc. of 80 per cent ethanol was heated under a reflux for 1 hour, diluted with ether, washed with water, and taken to dryness. The residue was recrystallized from ether and from acetone. The final product melted at 184–185°. Pregnenediol crystallized from acetone in heavy platelets which disintegrated *in vacuo* at room temperature. (Alcohol of crystallization was retained under these conditions.) The analytical sample was dried at 110° *in vacuo*.

Analysis—$C_{21}H_{34}O_2$. Calculated, C 79.19, H 10.76; found, C 79.30, H 10.81

Treatment of 35.8 mg. of the diacetate with 20 mg. of sodium hydroxide in 10 cc. of 95 per cent methanol at room temperature failed to effect complete hydrolysis within 19.5 hours.

The melting points of pregnenediol recorded in the literature are 172–176° (2), 170–174° (3), 174–176° (4). The first of these preparations was obtained from a fraction containing a contaminating substance "which rendered complete ,purification difficult." The second is described as "not pure."

Stability of Δ^5-Pregnenediol-3(β),20(α) towards Acid—2.8 mg. of pregnenediol were dissolved in 2 cc. of a solution of concentrated hydrochloric acid in 95 per cent ethanol (0.03 N) heated under a reflux for 2 hours and distributed between ether and water. The ether phase was washed free of acid, taken to dryness, and recrystallized from ether. The melting point (184–186°) of this product (2.8 mg.) remained unchanged upon admixture with the starting material.

A solution of pregnenediol in 90 per cent trichloroacetic acid which was kept at room temperature remained colorless for more than 1 hour (Rosenheim test).

Hydrogenation of Δ^5-Pregnenediol-3(β),20(α) Diacetate—A solution of 15.4 mg. of pregnenediol diacetate in 13 cc. of 95 per cent ethanol was shaken in an atmosphere of hydrogen (733 mm. of Hg, 26°) in the presence of 167 mg. of a palladium-calcium carbonate catalyst (1 per cent) (33). The reaction ceased after 12 minutes. The reduced product was recrystallized twice from methanol. Its melting point (168–169.5°) remained unchanged after admixture with allopregnanediol-3(β),20(α) diacetate (m.p. 168.5–170°) which had been prepared from allopregnanediol-3(α),20(α) by epimerization (5) and acetylation.

The reduced product was hydrolyzed with alcoholic sodium hydroxide as described above and the resulting material recrystallized from acetone. 10.0 mg. of heavy platelets were obtained which melted at 219–220° and at 218–220° upon admixture with an authentic specimen of allopregnanediol-3(β),20(α) melting at 217–220°.

Analysis—Sample dried at 110° *in vacuo*

$C_{21}H_{36}O_2$. Calculated, C 78.69, H 11.32; found, C 78.36, H 11.29

Oxidation of Δ^5-Pregnenediol-3(β),20(α)—20.4 mg. of pregnenediol were dissolved in 3.2 cc. of glacial acetic acid. A solution of bromine in the same solvent (about 23 mg. per cc.) was added until a faint yellow tint persisted (0.48 cc.). The mixture was treated with 10.8 mg. of chromium trioxide (in 0.36 cc. of 90 per cent acetic acid) for 4.5 hours at room temperature (31°). The excess of the oxidant was reduced with 0.2 cc. of alcohol. 120 mg. of zinc dust were added and the mixture was heated on a steam bath with stirring for 15 minutes. After being cooled, the liquid phase was separated, diluted with ether, and washed with water, cold sodium carbonate solution, and water. The ether residue, 18.4 mg.

of crystalline material, was recrystallized from dilute alcohol and from petroleum ether. The final product melted at 129–130.5° and at 129.5–131° upon admixture with an authentic specimen of progesterone melting at 129.5–131°. Upon resolidification all three samples melted at 123°.

Isolation of Δ^5-Androstenediol-3(β),17(α) Diacetate—Fraction 7 was recrystallized repeatedly from methanol and from petroleum ether. The final product (10.8 mg.) melted at 158–160°. Admixture with a sample of Δ^5-androstenediol-3(β),17(α) diacetate (m.p. 161.5–163°) which had been prepared from dehydroisoandrosterone (8, 9) raised the melting point to 159–161.5°.

Analysis—Sample dried at 80° *in vacuo*
$C_{23}H_{34}O_4$. Calculated, C 73.76, H 9.15; found, C 73.98, H 9.05

Fractions 8 and 9 were recrystallized from methanol and yielded 15.5 mg. of the same compound melting at 159–160.5°. The combined mother liquors of these three fractions furnished another 9.8 mg. (m.p. 154–159°). 5.7 mg. of the diacetate (m.p. 156–158.5°) were obtained from Fraction 6 after a lengthy series of recrystallizations. The identity of all these preparations was shown by mixed melting point determinations. Androstenediol diacetate has been reported to melt at 159.5°, uncorrected (9), and at 165–166°, corrected (8)

Hydrolysis of Δ^5-Androstenediol-3(β),17(α) Diacetate—15.5 mg. of androstenediol diacetate were hydrolyzed with 5 cc. of boiling ethanol (80 per cent) containing 32 mg. of sodium hydroxide in the manner described above. The resulting product upon recrystallization from ethyl acetate (8) melted at 178–182°. The melting point of a mixture with authentic Δ^5-androstenediol-3(β),17(α) (m.p. 179–181°) was 179–182°. The analytical specimen was recrystallized from a mixture of methanol, benzene, and petroleum ether (1:3:10) and dried at 110° *in vacuo.*

Analysis—$C_{19}H_{30}O_2$. Calculated, C 78.57, H 10.41, found, C 78.52, H 10.50

Repeatedly, partial fusion followed by resolidification was observed around 155° both with the isolated compound and the reference preparation. The melting point of androstenediol has been reported as 177–178°, uncorrected (9), and 182–183°, corrected (8).

Oxidation of Δ^5-Androstenediol-3(β),17(α)—12.4 mg. of androstenediol were brominated, oxidized, and debrominated in the manner described for pregnenediol. The neutral reaction product was recrystallized from dilute acetone and from mixtures of acetone and petroleum ether. It melted at 171–173.5° and at 171.5–173.5° upon admixture with a specimen of Δ^4-androstenedione-3,17 (m.p. 172.5–173.5°) which had been prepared from dehydroisoandrosterone (10). The three samples after resolidification melted at 144.5°, 144.5°, and 144° respectively. Two allotropic modifications of Δ^4-androstenedione-3,17 melting at 173–174°, corrected (10), and at 142–144° (34) have been described previously.

Isolation of Acetate of Compound A—Fraction 11 contained amorphous material that was separated from the crystalline product by virtue of its lesser solubility in hot methanol. After several recrystallizations from this solvent 9.1 mg. of fine needles were obtained which melted at 177.5–179.5°. Fraction 12 yielded another crop of crystals (5.3 mg.) melting at 176.5–179.5°. A mixture of both preparations melted at 178–180°.

Analysis—Sample dried at 80° *in vacuo*
$C_{27}H_{40}O_6$. Calculated, C 70.40, H 8 75; found, C 70.66, H 8.[illegible]6

Hydrolysis of Acetate of Compound A—10.2 mg. of Compound A triacetate, 32 mg. of sodium hydroxide, and 5 cc. of 80 per cent ethanol were heated under a reflux for 1 hour. The solution was taken up in ether, washed with water, and taken to dryness. The residue (7.5 mg.) was recrystallized from dilute and from absolute ethanol. Needle-shaped crystals were obtained which melted at 246–248° with decomposition.

Analysis—Sample dried at 110° *in vacuo*
$C_{21}H_{34}O_3$. Calculated, C 75.40, H 10 25; found, C 75 26, H 10.20

Isolation of Compound Ba—Fraction 14 was recrystallized repeatedly from methanol and from dilute methanol. 1.5 mg. of very long tetragonal plates were obtained, which showed a constant melting point at 232.5–234.5°.

Isolation of Acetate of Compound C—Fraction 15 contained resinous material which interfered with its purification by recrystallization. The fraction was dissolved in 5 cc. of benzene and passed through a column (80 × 5 mm.) of aluminum oxide. The adsorbent was washed with 7, 20, 20, 40, 45, 100, and 100 cc. of the same solvent, which eluted 65.4, 9.0, 5.1, 6.4, 2.6, 2.8, and 0.7 mg. respectively (Fractions 15-a to 15-g). Elution with 30 and 85 cc. of a 9:1 mixture of benzene and acetone, with 45 cc. of a 3:1 and with 100 cc. of a 1:1 mixture of these solvents, and with 50 cc. of acetone yielded 10.5, 3.7, 5.6, 3.9, and 1.4 mg. respectively (Fractions 15-h to 15-l). Most of these eluates failed to crystallize.

Fraction 15-a, the largest crystalline fraction, was recrystallized repeatedly from methanol, from acetone, and from a mixture of both. 4.2 mg. of very long tetragonal plates were isolated melting at 204–207°. Occasionally higher melting points (210°) were observed. Decomposition at these temperatures is indicated, since the resolidified melt although colorless showed a progressive lowering of the melting point on repeated fusions. A mixture of this substance with Compound Ba melted at 183–212°.

Analysis—Sample dried at 80° *in vacuo*

$C_{23}H_{34}O_5$.	Calculated.	C 70.74,	H 8.78
$C_{24}H_{36}O_5$.	"	" 71.25,	" 8.97
$C_{25}H_{38}O_5$	"	" 71.74,	" 9.15
	Found.	" 71.17,	" 8.96

4.5 mg. of less pure material (m.p. 200–204°) which had been obtained from the last five mother liquors were hydrolyzed with alcoholic sodium hydroxide as described above. The reaction product was recrystallized from absolute and dilute ethanol 1.5 mg. of rectangular platelets were obtained, which upon fairly rapid heating melted at 220–223° with decomposition.

SUMMARY

The benzene-soluble portion of non-ketonic compounds from the urine of a boy with adrenocortical carcinoma has been fractionated with digitonin. The precipitable material after acetylation and chromatographic analysis has yielded six crystalline compounds; *viz.*, (1) Δ^5-pregnenediol-3(β),20(α) diacetate, (2) a substance (m.p. 164–168°) regarded as a monoacetate of Δ^5-pregnenediol-3(β),20(α), (3) Δ^5-androstenediol-3(β),17(α) diacetate, (4) the triacetate (m p. 178–180°) of a triol $C_{21}H_{34}O_3$ (Compound A, m.p. 246–248°), (5) Compound Ba melting at 232.5–234.5°, and (6) an acetyl derivative (m.p. 204–207°) of Compound C (m.p. 220–223°). The parent substances of these acetates have not been encountered previously in human urine.

It is suggested that pregnenediol and androstenediol are derived at least in part from substances formed in the adrenal tumor. The chemical nature of possible precursors of these diols is briefly discussed.

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BIBLIOGRAPHY

1. Hirschmann, H., *J. Biol. Chem.*, **150**, 363 (1943).
2. Marker, R. E., and Rohrmann, E., *J Am. Chem. Soc* , **60**, 1565 (1938).

3. Marker, R. E., Tsukamoto, T., and Turner, D. L., *J. Am. Chem. Soc.*, **62**, 2525 (1940).
4. Marker, R. E., Crooks, H. M., Jr., and Wittbecker, E. L., *J. Am. Chem. Soc.*, **63**, 777 (1941).
5. Marker, R. E., Kamm, O., Wittle, E. L., Oakwood, T. S., Lawson, E. J., and Laucius, J. F., *J. Am. Chem. Soc.*, **59**, 2291 (1937).
6. Butenandt, A., and Schmidt, J., *Ber. chem. Ges.*, **67**, 2092 (1934).
7. Schoenheimer, R., and Evans, E. A., Jr., *J. Biol. Chem.*, **114**, 567 (1936).
8. Ruzicka, L., and Wettstein, A., *Helv. chim. acta*, **18**, 1264 (1935).
9. Butenandt, A., and Hanisch, G., *Z. physiol. Chem.*, **237**, 89 (1935).
10. Ruzicka, L., and Wettstein, A., *Helv. chim. acta*, **18**, 986 (1935).
11. Sobotka, H., Bloch, E., and Rosenbloom, A. B., *Am. J. Cancer*, **38**, 253 (1940).
12. Callow, N. H., *Biochem. J.*, **33**, 559 (1939).
13. Ruzicka, L., Goldberg, M. W., and Rosenberg, H. R., *Helv. chim. acta*, **18**, 1487 (1935).
14. Callow, N. H., Callow, R. K., and Emmens, C. W., *Biochem. J.*, **32**, 1312 (1938).
15. Crooke, A. C., and Callow, R. K., *Quart. J. Med.*, **32**, 233 (1939).
16. Wolfe, J. K., Fieser, L. F., and Friedgood, H. B., *J. Am. Chem. Soc.*, **63**, 582 (1941).
17. Marker, R. E., *J. Am. Chem. Soc.*, **61**, 944 (1939).
18. Marker, R. E., *J. Am. Chem. Soc.*, **61**, 1287 (1939).
19. Butenandt, A., Dannenbaum, H., Hanisch, G., and Kudszus, H., *Z. physiol. Chem.*, **237**, 57 (1935).
20. Callow, N. H., and Callow, R. K., *Biochem. J.*, **33**, 931 (1939).
21. Callow, N. H., and Callow, R. K., *Biochem. J.*, **34**, 276 (1940).
22. Hirschmann, H., *J. Biol. Chem.*, **136**, 483 (1940).
23. Beall, D , and Reichstein, T., *Nature*, **142**, 479 (1938).
24. Venning, E. H., and Browne, J. S. L., *Endocrinology*, **27**, 707 (1940).
25. Dorfman, R I., and Hamilton, J. B , *J. Biol. Chem* , **133**, 753 (1940). Dorfman, R. I., *Proc. Soc. Exp. Biol. and Med.*, **46**, 351 (1941).
26. Reichstein, T., and Shoppee, C. W., in Harris, R. S., and Thimann, K. V., Vitamins and hormones, New York, **1**, 345 (1943).
27. Heard, R. D. H., and Hoffman, M. M., *J. Biol. Chem* , **141**, 329 (1941).
28. Pincus, G., and Pearlman, W. H., *Endocrinology*, **31**, 507 (1942).
29. Mamoli, L., Koch, R., and Teschen, H., *Z. physiol. Chem.*, **261**, 287 (1939).
30. Frank, R. T., Klempner, E., Hollander, F., and Kriss, B., *Endocrinology*, **31**, 63 (1942).
31. Girard, A., and Sandulesco, G., *Helv. chim. acta*, **19**, 1095 (1936).
32. Schoenheimer, R., and Dam, H., *Z. physiol. Chem.*, **215**, 59 (1933).
33. Busch, M., and Stoeve, H., *Ber. chem. Ges.*, **49**, 1063 (1916).
34. David, K., *Acta brev. Neerl.*, **5**, 108 (1935).

STUDIES ON HUMAN BLOOD PROTEINS*

I. THE ISOLEUCINE DEFICIENCY OF HEMOGLOBIN

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(Received for publication, October 18, 1944)

The need and importance of securing adequate substitutes for human blood protein preparations now so extensively and successfully employed in the treatment of traumatic or hemorrhagic shock are obvious. Our approach to this problem is based on the assumption that the preparation of suitable substitutes might be facilitated by an accurate knowledge of the amino acid make-up of the blood proteins. The study of human hemoglobin was undertaken first because it can be isolated in a well defined crystalline form. Accordingly, crystalline hemoglobin was prepared from human red blood cells and analyzed chemically for tyrosine, cystine, alanine, and the ten amino acids currently considered necessary for physiological maintenance of the human adult (1). This study, the details of which will be reported later, revealed that the isoleucine N constitutes approximately 0.5 per cent of the total human hemoglobin N.

This interesting finding prompted us to check the chemical result by bioassay in the rat which has been shown by the use of amino acid mixtures to require dietary isoleucine for both growth (2) and maintenance of adult weight (3). The results of these experiments are reported here and confirm the deduction made from the analytical data, namely, that a diet in which human red blood cell histone comprised the protein moiety would fail to support growth in the immature rat and weight balance of the adult animal unless supplemented with isoleucine. Since supplementation of the deficient diet with *d*(−)-isoleucine failed to support normal growth in the immature rat, it is apparent that only the *l* variety of this amino acid is available to the animal.

EXPERIMENTAL

Animals—These observations were made on rats from a hybrid colony of albino and hooded Norwegian rats that have been in use in this labora-

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tory for some years. Individual litters were divided so as to obtain an equitable distribution of weight and sex in the deficient and control groups. During the experiments, they were kept in individual cages which were not designed in such a way as to prevent coprophagy. The weight measurements were made weekly.

Preparation of Diets—The composition of the diets is shown in Table I. The protein moiety of the diet was composed of a tryptophane and cystine reinforced acid hydrolysate of crystalline human hemoglobin prepared as described below. In order to maintain the protein level of the diets constant, a reduction was made in the amount of hydrolysate added to the diet proportionate to the amount of amino acid supplementation. Owing to uncertainties regarding the B complex vitamins, brewers' yeast was used instead of a mixture of the synthetically available components of this vitamin group. The amount of isoleucine introduced into the diet in this manner is apparently negligible with respect to the needs of the rat. Except when indicated all of the animals were fed *ad libitum* and the amount of food consumed daily recorded.

TABLE I

Composition of Diets

Diets	A	B	C	D
	gm.	gm.	gm.	gm
Human hemoglobin	15.5	14.1	14.8	14.1
Brewers' yeast	4.0	4.0	4.0	4.0
Sucrose	14.1	14.1	14 1	14.1
Starch	39.3	39.3	39.3	39.3
Agar	2.0	2.0	2.0	2 0
Crisco	18.0	18.0	18.0	18.0
Cod liver oil substitute*	4.7	4.7	4.7	4.7
Salt mixture†	2.0	2.0	2.0	2.0
l(−)-Tryptophane	0.23	0.23	0.23	0.23
l(−)-Cystine	0.17	0.17	0.17	0.17
dl-Isoleucine	0	1.40	0.7	0
d(−)-Isoleucine	0	0	0	1.4
	100.0	100.0	100.0	100.0

* Mead Johnson and Company.

† The salt mixture employed had the following composition (measured in gm.): NaCl 18.9, $CaHPO_4$, anhydrous, 25.0, $MgSO_4$, anhydrous, 6.86, $KHCO_3$ 44.4, KCl 2.88, Fe^{III} citrate, U. S. P., 2.21, $CuSO_4$, anhydrous, 0.24, $MnSO_4$, anhydrous, 0.15, KI 0.015, NaF 0.03.

Preparation of Human Hemoglobin Hydrolysates—Human hemoglobin was prepared from human red blood cells by the method of Zinoffsky (4).

10 liters of red blood cells, processed 1 liter at a time, yielded 700 gm. of crystalline hemoglobin which contained 13.96 per cent nitrogen, uncorrected for moisture and ash.

500 gm. of the hemoglobin were hydrolyzed under a reflux for 24 hours with 2 liters of 20 per cent sulfuric acid. After cooling, the hydrolysate was alkalinized by the slow addition of a solution containing 310 gm. of calcium oxide (technical) in 2 liters of water. The mixture was stirred well and resulted in the evolution of ammonia. After standing overnight it was filtered and the calcium sulfate cake washed by resuspension in 2 liters of warm water. The major portion of the pigments remained adsorbed on the calcium sulfate. The combined filtrate and washings were concentrated *in vacuo* at 50–60° to approximately 1 liter. The ammonia-free concentrate was now made neutral to litmus with 50 per cent sulfuric acid, cooled under the tap, and the resulting calcium sulfate filtered off. The protein equivalent (N × 7.2) of the resulting filtrate was estimated from a micro-Kjeldahl analysis (5) of a suitable aliquot. For use in the diet, this product was further concentrated *in vacuo* to approximately 400 cc. and supplemented with 1.5 per cent *l*(−)-tryptophane and 1.0 per cent *l*(−)-cystine of the protein equivalent.

The *l*(−)-tryptophane, *l*(−)-cystine, and *dl*-isoleucine used for supplementation of the diets were obtained from Merck and Company, Inc., and their purity checked in this laboratory. The *d*(−)-isoleucine used in these experiments was kindly prepared for us by Hoffmann-La Roche, Inc., by enzymic resolution of the racemate. This product was characterized by the nitrogen content and the optical activity of a 3.1 per cent aqueous solution.

$C_6H_{13}O_2N$ Calculated, N 10.68; found N 10.65
Locquin (6), $[\alpha]_D^{20} = -10.55°$, found $[\alpha]_D^{20} = -12.25°$

Results—In the preliminary experiment, eight immature rats were placed on Diet A. The weight losses caused by Diet A (Fig. 1) revealed its nutritional inadequacy. The animals were then separated into four groups and each group was fed one of four test diets prepared by supplementing Diet A singly with the racemic forms of isoleucine, leucine, valine, and threonine. The results of this experiment made it evident that supplementation with isoleucine alone permitted resumption of growth in the animals. This initial observation was checked by the fact that the animals fed the threonine-, valine-, or leucine-supplemented diets also gained weight when given the isoleucine-supplemented diet, and further confirmed by the data on five additional animals which are not included in Figs. 1 to 4. These experiments demonstrated conclusively that Diet A, and hence human red blood cell globin, is deficient in isoleucine. Feeding of the deficient diet to rats of different ages demonstrated that isoleucine is required for growth by the immature rat and for maintenance of weight by the adult rat (Fig. 2). Representative growth curves of another group of animals which were paired fed indicate that a 50 per cent reduction in the isoleucine supplement, Diet C, results in a proportionate loss of nutritional value of the diet (Fig. 3).

In order to test the availability of *d*(−)-isoleucine some of the animals on Diet A or B were changed to Diet D. The growth curves of these animals (Fig. 4) show that a slight growth stimulation is obtained from Diet D. This might be due to an actual slight utilization of the *d* form by the rat or the presence of traces of the *l* form in the resolved product. The latter probability is made more plausible by the observation that animals on Diet B lost weight when placed on Diet D.

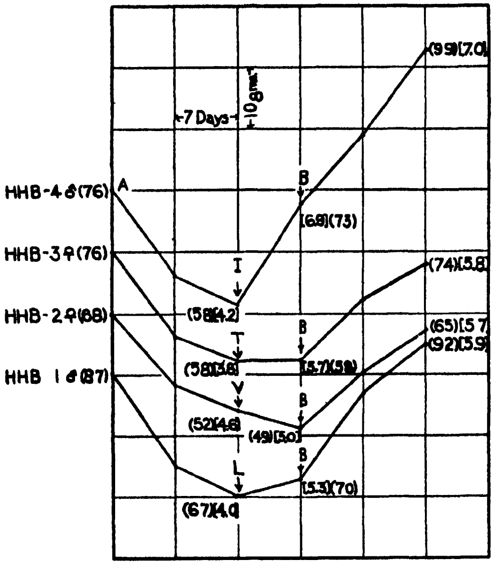

FIG. 1. The effect of amino acid supplements on the nutritive value of hydrolyzed human hemoglobin. All the animals were initially fed Diet A, which was supplemented singly with 1.4 per cent of the racemates of isoleucine (*I*), threonine (*T*), valine (*V*), and leucine (*L*) at the points indicated by the arrows After 1 week, the animals were all fed Diet B, to the end of the experiment The figures in parentheses denote the weight of the animal in gm., those in brackets the average daily food consumption for each feeding period

Comment

Experiments on the nutritional adequacy of human plasma proteins reported in abstract by Stare and associates (7) during the course of this

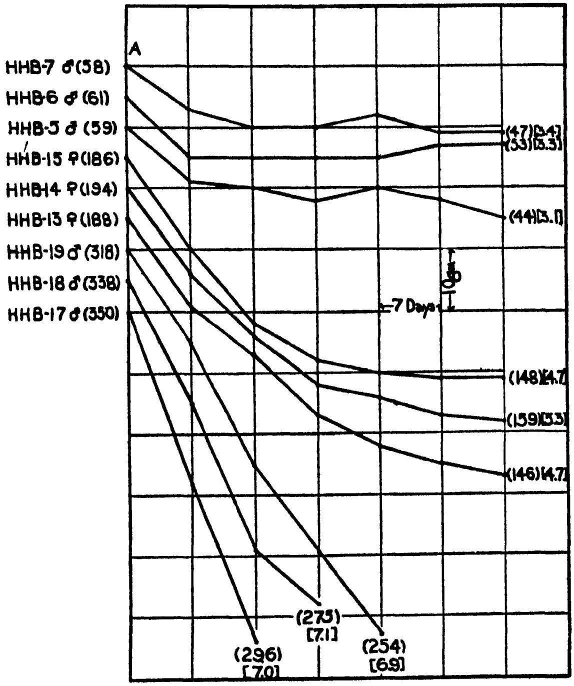

FIG. 2. The effect of the isoleucine-deficient diet on rats of various ages. All animals were fed Diet A throughout. The figures in parentheses denote animal weight, those in brackets the average daily food intake

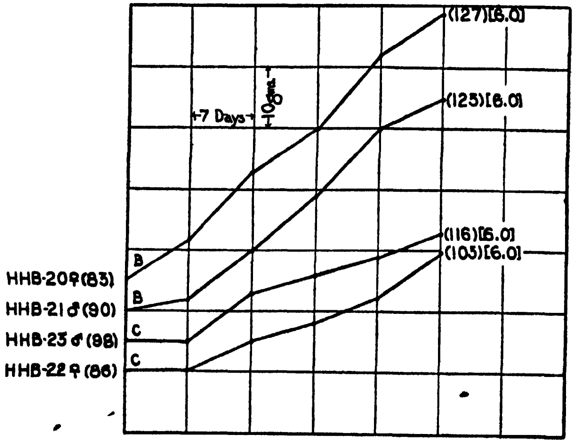

FIG. 3. Growth effect of one-half reduction of the isoleucine supplement. Animals were fed Diet B or C as indicated. The figures in parentheses denote weight of animals, those in brackets the average daily food intake.

study show that the plasma proteins are also poor in isoleucine. The fact that their animals gained an average of 0.9 gm. daily on the plasma protein diets would indicate the isoleucine content of these proteins to be much greater than that of hemoglobin. It would appear therefore that only small amounts of isoleucine are required for the regeneration of human plasma proteins and hemoglobin. The rapid recovery from traumatic or hemorrhagic shock achieved by the administration of human blood protein preparations would seem to corroborate this interpretation of the experimental data.

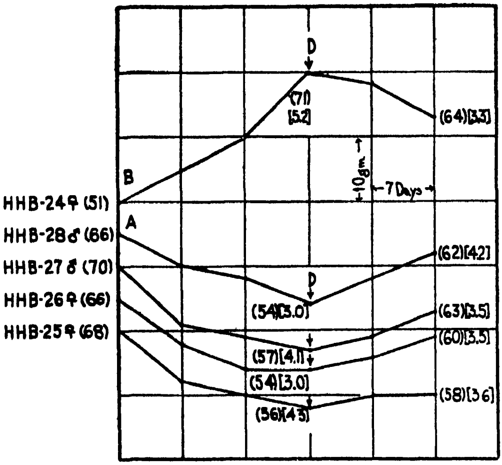

FIG. 4. The availability of *d* (—)-isoleucine for growth in the rat Animals were fed Diet A or B as indicated and changed to Diet D at the arrow The figures in parentheses denote the animal weight in gm., those in brackets the average daily food intake.

Quite apart from the immediate purpose of the study, these findings afforded us an opportunity to test the biological availability of *d*(—)-isoleucine. Although our data on this point are not so conclusive as might be desired, they would appear to confirm the findings of Rose (8) that *d*(—)-isoleucine is not utilized for growth by the rat.

SUMMARY

Human hemoglobin has been shown by bioassay in the rat to be deficient in isoleucine. Consideration of current knowledge suggests that only small amounts of isoleucine are required for the formation of hemoglobin and the plasma proteins. *d*(—)-Isoleucine does not appear to be available for growth in the immature rat.

BIBLIOGRAPHY

1. Holt, L. E., Jr., and Albanese, A. A., *Tr. Assn. Am. Physn.*, *in press.*
2. Womack, M., and Rose, W. C., *J. Biol. Chem.*, **116,** 381 (1936).
3. Burroughs, E. W., Burroughs, H. S., and Mitchell, H. H., *J. Nutr.*, **19,** 363 (1940).
4. Zinoffsky, C., *Z. physiol. Chem.*, **10,** 16 (1886).
5 Meeker, E. W., and Wagner, E. C., *Ind. and Eng. Chem., Anal. Ed.*, **5,** 396 (1933).
6 Locquin, R., *Bull Soc. chim.*, **1,** 595, 601 (1907).
7 Stare, F. J , Hegsted, D. M., and McKibbin, J. M., *Federation Proc.*, **3,** 96 (1944).
8. Rose, W. C , *Physiol. Rev* , **18,** 109 (1938).

THE ACTION OF TRYPSIN ON NATIVE AND DENATURED PROTEINS

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It has been demonstrated by several authors that ovalbumin (1), pseudoglobulin (2), serum albumin (2, 3), lactoglobulin (4), and other proteins are *attacked by trypsin less readily in their native state* than when they are denatured. Experiments carried out in our own laboratory revealed, however, that the tryptic hydrolysis of fibrinogen and of myosin proceeds *at the same rate before and after their denaturation* (5). In these as well as in most of the previously mentioned experiments, commercial trypsin or insufficiently purified preparations of the enzyme have been used. On account of the low efficiency of such preparations rather large quantities of enzyme were required; their amount varied from 5 per cent (4) to 80 per cent (2) of the proteins examined. The commercial trypsin (Merck) used in our previous experiments (5) contained approximately 0.06 trypsin unit (6) $[\text{T. U.}]^{\text{Kas. F.}}$ per gm. of nitrogen. The possibility could not be excluded that the opposite results, obtained with fibrinogen and myosin on the one hand and with albumins and globulins on the other hand, might be due to the effect of inhibitors or other impurities present in the trypsin preparations. We have repeated therefore our experiments with trypsin, prepared according to Northrop (7). Owing to the greater efficiency of the purified enzyme, we were able to reduce the amount of the enzyme to 0.5 to 2.0 per cent of the proteins examined; at the same time the duration of our previous experiments was reduced substantially, *e.g.* from 24 hours to 30 minutes. The possibility that labile proteins are denatured by the buffer solution or by toluene at 38° is reduced in such short experiments.

EXPERIMENTAL

Preparation of Substrates—Crystalline ovalbumin was prepared from hen's eggs (8), pseudoglobulin and euglobulin from sheep serum (9), collagen from ox bones (10), myosin from fresh rabbit muscle (11); fibrinogen was precipitated from blood plasma of the ox by NaCl (12). The precipitate was dissolved in water and reprecipitated by mixing with 2 volumes of a saturated solution of NaCl, containing 0.3 per cent of potassium oxalate. Ovalbumin and pseudoglobulin were purified by dialysis against distilled water.

Preparation of Trypsin—Trypsinogen was extracted from fresh ox pancreas with sulfuric acid according to the method of Kunitz and Northrop (7). Chymotrypsinogen was removed by the addition of ammonium sulfate; trypsinogen, precipitated by further addition of ammonium sulfate, was reprecipitated, washed with a saturated solution of magnesium sulfate in 0.02 N sulfuric acid (7), and dissolved in a small quantity of water. This solution was activated by the addition of borate buffer solution and by keeping in the ice box at pH 8 for several days (7). The Trypsin I solution used in Experiments 1 to 6 (Table I) contained 5.9 mg. of protein N per ml. with an activity of 0.17 $[\text{T. U.}]^{\text{Kas F}}$ (6); Trypsin II used in Experiments 7 to 12 contained 1.8 mg. of protein N and 0.08 $[\text{T. U.}]^{\text{Kas F}}$ per ml. respectively.

Determination of Rate of Hydrolysis—5 ml. of a solution containing 2.5 per cent of the native protein and 1 per cent of NaCl were put in each of eight small flasks. Since euglobulin and collagen do not dissolve in saline solution, 2.5 per cent suspensions of these proteins were used. 2 ml. of a 0.2 M solution of phosphate buffer, pH 8.0, and 1.0 ml. of water were added to each flask. Four of these flasks were weighed and then kept in a boiling water bath for 30 minutes. The coagulated protein was finely ground and water was added to its suspension in order to replace the water lost by evaporation. All flasks were then warmed to 38°; 1 drop of toluene and 0.05 ml. of the solution containing Trypsin I were added in Experiments 1 to 6; 0.17 ml. of Trypsin II was added in Experiments 9 to 11; and 0.05 ml. in Experiments 7, 8, and 12. At the times *t* (recorded in Table I) 2 ml. of a neutralized 30 per cent solution of formaldehyde and 0.1 ml. of 1 per cent phenolphthalein were added to each of the protein samples and the rate of hydrolysis was determined by titration with 0.1 N NaOH. The results obtained from formol titrations are shown in Table I.

Results

The experiments recorded in Table I reveal the different behavior of globular proteins (ovalbumin, serum globulin) on the one hand and fibrous proteins (fibrinogen, myosin) on the other. Fibrous proteins were hydrolyzed by trypsin at the same velocity before and after denaturation of the protein, while globular proteins were attacked by trypsin much more rapidly after denaturation, although only the surface of the coagulated particles could be accessible to the enzyme. No hydrolysis or a trace only took place in the experiments with *native* globular proteins in the course of the first 30 minutes. But even after 4 or 24 hours the degree of hydrolysis of native globular protein remained far below that of denatured protein. Collagen, although a fibrous protein, is resistant to trypsin. We attribute this to the insolubility of collagen in water and to the fact that coarse particles of the protein were used in our experiments. Heating in the water bath brought about a swelling of these particles and partial dissolution, *i.e.* formation of gelatin.

TABLE I

Hydrolysis of Native and of Denatured Proteins by Trypsin at pH 8

The third column of the table indicates the *quantity* of 0.1 N NaOH, required for formol titration at the beginning of the experiment; the figures in the last three columns indicate the *excess* of 0.1 N NaOH required

Experiment No	Protein	Quantity of 0.1 N NaOH, $t = 0$	Excess 0.1 N NaOH		
			$t = 30$ min	$t = 4$ hrs	$t = 24$ hrs.
		ml	*ml*	*ml*	*ml*
1	Ovalbumin, native	1.31	0.17	0.22	0.32
	" denatured	1.11	0.25	0.48	1.00
2	Pseudoglobulin, native	2.16	0.04	0.11	0.28
	" denatured	1.70	1.07	1.16	2.04
3	Collagen, native	0.73	0.07	0.19	0.50
	" denatured	0.80	0.18	0.68	3.16
4	Fibrinogen, native	1.26	0.81	1.39	2.13
	" denatured	0.87	0.78	1.67	2.42
5	Myosin, native	1.94	1.28	1.72	2.74
	" denatured	1.58	0.80	1.78	2.79
6	Casein (Merck)	1.03	1.83	2.19	3.10
7	Pseudoglobulin, native	1.50	0.10	0.15	0.30
	" denatured	1.25	0.40	1.05	1.55
8	Euglobulin, native	1.32	0.10	0.28	0.93
	" denatured	1.19	0.25	0.96	1.59
9	Ovalbumin, native	2.17	0.0	0.10	0.30
	" denatured	2.17	0.23	0.63	1.15
10	Collagen, native	1.60	0.10	0.18	0.29
	" denatured	1.73	0.29	0.79	1.02
11	Fibrinogen, native	1.96	0.46	0.84	1.18
	" denatured	2.05	0.50	0.95	1.31
12	Myosin, native	3.56	0.10	0.54	1.76
	" denatured	3.52	0.09	0.66	1.63

DISCUSSION

The resistance of native proteins to trypsin had been ascribed originally to the presence of an antitryptic factor in the blood serum (13) or in raw egg white (14). It is, however, extremely improbable that such a factor would be present in all fractions of the blood serum (*i.e.* serum albumin, pseudoglobulin, and euglobulin) at the same time (5).

Linderström-Lang (15) and Lundgren (16) attribute the resistance of native globular proteins to the fact that the atomic groups serving as points of attack for the enzyme are inside the globular molecule and inaccessible to the proteinase. Denaturation, which apparently involves an unfolding of the closely packed peptide chains (17, 18), renders these groups accessible to the enzyme. The view of Linderström-Lang (15) is corroborated by our own experiments, for no steric hindrance can be expected in fibrous proteins such as fibrinogen or myosin, whose peptide chains are either expanded or only slightly folded.

These results are probably of some importance for the nutrition of man. It is well known that the proteins of foodstuffs are attacked at first by pepsin at pH 1 to 2; the old experience that native and denatured proteins are hydrolyzed by pepsin at the same rate was confirmed by experiments carried out in our laboratory. But in patients suffering from gastric achylia, when no digestion occurs in the stomach, the raw proteins of the food enter the intestines and undergo there the action of trypsin. According to our experiments one would expect that raw *globular* proteins resist the action of trypsin, while *fibrous* proteins ought to be rapidly hydrolyzed. Actually Talarico (19) showed some time ago that raw eggs are resistant to trypsin, while boiled eggs are rapidly hydrolyzed; raw meat, however, is digested just as rapidly as boiled meat. Results of similar experiments carried out in our laboratory agree with those of Talarico (19), and so details of these experiments may be omitted. We attribute the digestion of raw meat by trypsin to its high content of fibrous proteins.

Text-books of biochemistry (20) or of therapeutics (21) used to recommend raw protein food, *e.g.* raw meat, which is more easily digested than boiled food. This is contradictory to the results of our experiments. According to these experiments boiling may raise the digestibility of raw protein food, but it never reduces it.

SUMMARY

1. While globular proteins such as ovalbumin or serum globulin are hydrolyzed by trypsin very slowly in their native state, fibrous proteins such as fibrinogen or myosin are hydrolyzed by trypsin at the same rate before and after denaturation. The susceptibility of native fibrous proteins to the attack of trypsin is attributed to the expanded configuration of their peptide chains, which renders their peptide bonds accessible to the enzyme.

2. The digestibility of raw protein food (meat, eggs, milk) by trypsin is not superior to that of boiled food.

BIBLIOGRAPHY

1. Calvery, H. O., *J. Biol. Chem.*, **102**, 73 (1933).
2. Bernheim, F., Neurath, H., and Erickson, J. O., *J. Biol. Chem.*, **144**, 259 (1942).

3. Giedroyć, W., Janicki, J., and Cichocka, J., *Enzymologia*, **5**, 81 (1938)
4. Linderström-Lang, K , and Jacobsen, C. F., *Compt.-rend. trav Lab. Carlsberg*, **24**, No 1 (1941).
5. Haurowitz, F , Tunca, M., and Yurd, N , *Bull. Fac. Méd. Istanbul*, 3126 (1943).
6 Northrop, J. H., and Kunitz, M , *J Gen. Physiol.*, **16**, 313 (1933)
7. Kunitz, M , and Northrop, J. H., *J. Gen. Physiol.*, **19**, 991 (1936)
8. Sörensen, S P. L , and Höyrup, M., *Z. physiol. Chem* , **103**, 16 (1918).
9 Haurowitz, F , Sarafyan, K , and Schwerin, P , *J. Immunol* , **40**, 391 (1941).
10. Strauss, E , in Abderhalden, E., Handbuch der biologischen Arbeitsmethoden, Berlin, **1**, 560 (1922)
11 Bayley, K , *Biochem J* , **36**, 122 (1942).
12 Hammarsten, O , quoted by Thierfelder, H , Handbuch der physiologisch- und pathologisch-chemischen Analyse, Berlin, 461 (1924)
13 Landsteiner, K , *Zentr Bakt* , **27**, 357 (1900); quoted by Abderhalden, E , Biochemisches Handlexikon, Berlin, **3**, 493
14 Cohn, E. W , and White, A , *J Biol. Chem* , **109**, 169 (1935)
15 Linderstrom-Lang, K , Annual review of biochemistry, Stanford University, **8**, 45 (1939)
16 Lundgren, H P , *J Biol Chem* , **138**, 293 (1941).
17 Wu, H , *Chinese J. Physiol* , **5**, 321 (1931).
18 Haurowitz, F , and Marx, F , *Kolloid-Z* , **74**, 208 (1936), **77**, 65 (1936)
19 Talarico, J , *Compt rend. Soc biol* , **68**, 662, 932 (1910), **69**, 324 (1910)
20 Cameron, A T , A textbook of biochemistry, London, 6th edition, 387 (1942)
21 Frank, R , Moderne Therapie, Berlin, 180, 186 (1934).

ON THE SILVER COMPOUND OF CASEIN IN ALKALINE MEDIUM

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(Received for publication, November 8, 1944)

This paper continues a series of publications which, starting in 1936 from a study of the biuret reaction, were carried out in collaboration with Jesserer (1).

As the existence of definite compounds of casein and other proteins and some peptones in an alkaline medium had been shown for copper and gold as well as for nickel and cobalt, it was logical, on account of the position of silver in the periodic system of elements, to anticipate that silver compounds might be formed under similar conditions. The investigation showed, however, that in the case of silver several complicating factors have to be considered, which play no rôle with the other metals mentioned.

From the extensive literature on silver-protein compounds we may select the important work of Pauli and Matula (2), Kruyt and Boelman (3), and Carroll and Hubbard (4); most of the experiments were carried out with serum albumin and gelatin, a few also with casein, with addition of silver nitrate or silver oxide. Increasing amounts of silver are bound with increasing concentrations of the salt or oxide, with a tendency to reach a maximum value. The effect of increasing the pH in gelatin experiments (4) appears to be an increase of the combining capacity of Ag ions per unit weight of gelatin; there is a minimum, not zero, value at the isoelectric point. The present paper deals exclusively with the experimental conditions described in the former papers listed (1) and with the properties of the silver compounds as compared with those of copper, nickel, and cobalt.

The substrate used was casein (Hammarsten, Merck), and in later experiments casein highest purity (City Chemical Corporation, New York). When these products are dissolved in 3 per cent NaOH and the metal salt is added, then, in the case of copper, etc., the metal hydroxides precipitated can be separated quantitatively by filtration from the solution of the metal-protein compound. In the case of silver, on the other hand, this separation proves inadequate because part of the silver oxide formed is protected by the protein from precipitation by the alkali. Repeated filtrations on hardened filter paper (Schleicher and Schüll), as well as repeated centrifuging followed by filtration, proved insufficient to avoid the presence of some Ag_2O.

3 gm. of casein are dissolved in 30 cc. of 30 per cent NaOH, 3 gm. of

metal salt in water are added, and the volume is adjusted to 300 cc. (3 per cent NaOH). The solution is allowed to stand (or eventually is shaken or aerated at the water pump) for about 24 hours; it is then centrifuged (1 to 2 hours) and filtered on hardened paper through Silex (Baker Chemicals). The filtrate is deep brown. In experiments with copper there is present in the protein filtrate an average of 58 mg. of Cu for 0.5 gm. of casein, *i.e.* 50 cc. of solution. The corresponding figure for silver should be 98.5 mg., if one assumes that silver occupies the same places as copper, probably attached to the nitrogen atoms of the peptide bonds. The figure found with silver, however, is always somewhat *higher* (110 to 150 mg. for 0.5 gm. of casein).

It has been shown in previous papers that, on *acidification* of the alkaline solution, the copper and the nickel compounds are immediately decomposed and the metal is found quantitatively as ion in the filtrate from the precipitated protein (casein); when the alkaline reaction is reestablished (before filtration), the metals mentioned again recombine in their previous positions in the peptide chains. The cobalt as well as the gold compounds are more resistant to acid. In the case of silver, acidification detaches the metal from the protein, just as with copper, but the silver oxide in colloidal solution is now precipitated along with the casein. Thus, a quantitative separation of the silver oxide and the silver bound in the alkaline medium to the protein is accomplished (Table I). If (before filtration) the solution is again made alkaline, the silver, like copper, returns to its former positions. The figures in Experiment B, Table I, are in good agreement with the average (98.5 mg. for 0.5 gm. of casein) to be expected for silver. But here another complicating factor comes into play.

TABLE I

Silver per 0.5 Gm. of Casein

Experiment A	Experiment B
mg.	*mg.*
120.1	99.4
149.0	102.1
112.3	100.2

Experiment A, direct determination (Volhard) in 50 cc. after centrifuging, filtering on Silex, and ashing with H_2SO_4-HNO_3 (Neumann) mixture; Experiment B, after acidification of solution in Experiment A with diluted H_2SO_4, filtering from the protein precipitated, and ashing.

The silver compound of casein in alkaline solution is more *unstable* than, *e.g.*, the copper compound in respect to *aging* of the solution as well as to an *increase in temperature*. It must be assumed that the silver which separates, slowly while standing and faster at a higher temperature, forms the oxide in the alkaline medium and is thus, on acidification, precipitated with the casein in increasing quantities, the quantity of silver found in the filtrate will therefore *diminish* (Table II). With analogous copper and nickel compounds these aging and temperature effects do not appear. With silver too, a *short* heating (*e.g.*, ¾ hour at 70°) causes no splitting off of the metal.

When silver does *not* form compounds with the colloidal matter in alkaline solution, as *e.g.* with starch or agar-agar (1 per cent solution in 3 per cent NaOH, 1 gm. of $AgNO_3$ to 1 gm. of starch or agar-agar), the sample standing for a day in the ice box yielded, after centrifuging and decanting, for 50 cc. 28.1 mg. of Ag, the analogous figure at room temperature being 54.1 mg. of Ag; thus we see that, with the effect of temperature on Ag_2O alone, the formation of *larger* particles of Ag_2O is favored by *lower* temperature and accordingly *less* silver will be found in the filtrate.

TABLE II

Silver (Volhard) per 0.5 Gm. of Casein Found in Protein Filtrate after Acidification

	Sample 1	Sample 2
	mg.	mg.
Sample stood 1 day after preparation at room temperature, 20°	100.2	100.2
“ “ 2 days “ “ “ “ “ 20°	82.5	
“ “ 4 “ “ “ “ “ “ 20°	70.9	82.9
“ “ 5 “ “ “ “ “ “ 20°		69.1
“ “ 11 “ “ “ “ “ “ 20°		34.6
“ “ 1 day “ “ “ “ “ (about 30°)	95.6	
Sample stood 3 days after preparation in ice box	74.5	
“ “ 3 “ “ “ at room temperature (about 30°)	43.5	

Shaking, for 24 hours, will give with the silver-protein solution different figures, according to the prevalence of one of two opposite effects: the temperature effect (see above), which has the tendency to *lower* the amount of silver in the acidified protein filtrate, and the effect of mere shaking, which diminishes (as special tests showed) the protective ability of the protein to hold Ag_2O in solution. This tendency will *increase* the amount of silver found in the filtrate. The concurrence of these tendencies will, of course, give rise to variable experimental results.

It was furthermore found that, in the case of *cobalt* compounds, shaking the samples (*e.g.*, 1 gm. of casein, 1 gm. of $CoSO_4$ in 100 cc. of 3 per cent NaOH) through 24 hours will greatly *increase* the quantity of cobalt taken up by the protein. Instead of the average value of about 18 mg. per 0.5

gm. of casein, previously found, corresponding to a third of the quantity of or 3 times the "space" taken by silver or copper, twice this quantity or more may combine with the protein.

It could be assumed that the cause of the lack of stability of the Ag-protein compounds was a specific disintegrating effect of silver on the protein, which would slowly lower its ability to combine, an action not shared by copper, etc. If this were true, the nitrogen in the casein filtrates ought to increase at a faster rate in the silver as compared with the copper samples. This, however, was *not* the case (Table III). There is, approximately, the same rate of disintegration in the silver as in the copper samples.

Replacement of Ag by Cu, Ni, or Co and of Cu, Ni, or Co by Ag—The protein solutions, after addition of the first metal salt, stand for 24 hours (eventually with shaking in the case of Cu or Ni); they are filtered on Silex and, in the case of Ag or Co, centrifuged for 1 to 2 hours. To the filtered and, eventually, centrifuged solutions, the second metal salt is added (1 gm. of salt per 100 cc.) and, after 24 hours, the solution is filtered and eventually centrifuged once more. The solution is then acidified with dilute H_2SO_4 and filtered from the protein precipitate. In the final filtrate a definite amount (generally 50 cc.) is ashed with H_2SO_4-HNO_3 (Neumann) mixture; the silver is precipitated and weighed as AgCl; the copper is precipitated as CuS and weighed as CuO; the nickel is precipitated and weighed as dimethylglyoxime compound (5); the cobalt is determined by iodine-thiosulfate titration (6). All the figures given are referred to 0.5 gm. of casein.

TABLE III

Mg. of Nitrogen (Kjeldahl) in Protein Filtrate per 0.5 Gm. of Casein

	Copper		Silver	
	Sample 1	Sample 2	Sample 1	Sample 2
Immediately after filtration	30.6	37.0	31.1	44.1
After 4 days	45.4		38.2	
" 5 " . . .	46.2			
" 7 " ..	47.5	60.7	47.0	66.4

When Ag is first present, it will be displaced to a large extent by Cu (added as $CuSO_4 \cdot 5H_2O$); *e.g.*,

(*a*) Ag in acid filtrate at start, 99.4 mg ; *after* addition of $CuSO_4 \cdot 5H_2O$, 4 days after start, Ag 2.8 mg., Cu 48.0 mg.

(*b*) Ag in acid filtrate at start, 108.8 mg.; *after* addition of $CuSO_4 \cdot 5H_2O$, 4 days after start, Ag 9.0 mg., Cu 53.5 mg.

The total of metal taken up, calculated as silver, in these two experiments, is 84.3 and 100.0 mg. of "Ag" respectively. The average figures for these sums were rather lower than 98 mg. of Ag (the figure for Ag alone) on account of the depressing effect of aging of the solutions on the silver value (see above). If Ni (as $NiSO_4$) is added as the second metal, it enters the peptide chains only to a limited amount and little silver is displaced; *e.g.*, Ag in the acid filtrate (*a*) at start, 94.0 mg.; *after* addition of $NiSO_4$, 4 days after start, Ag 77.9 mg., Ni 1.6 mg.; (*b*) at start, 108.8 mg.; *after* addition of $NiSO_4$, 4 days after start, Ag 81.0 mg., Ni 8.2 mg. The sums are 83.3 and 111.6 mg. of "silver" respectively. They are calculated on the assumption that 1 atom of Ni will displace 2 atoms of silver, just as is the case with copper (see former paper (1)). The second example given has an exceptionally high total sum, the average being around 86 mg. Cobalt, added as the second metal as $CoSO_4 \cdot 7H_2O$, will displace varying amounts of silver; the total of metal taken up is calculated on the assumption that 1 atom of Co will displace 3 atoms of silver (just as with copper (1)); *e.g.*,

(*a*) Ag in acid filtrate at start, 92.9 mg ; *after* addition of $CoSO_4 \cdot 7H_2O$, 4 days after start, Ag 56.2 mg., Co 2.7 mg.

(*b*) Ag in acid filtrate at start, 93.7 mg ; *after* addition of $CoSO_4 \cdot 7H_2O$, 4 days after start, Ag 15.8 mg , Co 13.2 mg.

The totals are 71.0 mg. and 86.9 mg. of "silver" respectively. Most of the values thus found for the sums were low, not only on account of the aging effect for silver, but also because the solutions were generally centrifuged a second time, with *both* metals present. This second centrifuging was omitted in the second example cited.

When silver (as $AgNO_3$) is added to the copper, as well as to the nickel- and cobalt-casein compound solutions, in all three cases the metal first present will be displaced by silver only to a small extent. As an average of the experiments 54.0 mg. of Cu and 7.8 mg. of Ag were found in the final solutions, the average sum, calculated as silver, being 100.0 mg. As silver is here added last, there is no effect of aging to be expected. The average figure for Cu alone was 58.0 mg.

When copper was added to nickel- or cobalt-protein solutions, there was no displacement of the metals first present, but the copper occupied the places in the peptide chains not occupied by nickel or cobalt; that is, one-half and two-thirds respectively.[1] With silver added, there was generally a displacement to a small degree and moreover the filling out of the empty places, as with Cu; *e.g.* 23.1 mg. of Ni and 63.8 mg. of Ag were found in a final solution, while the average figure for Ni alone was 26 mg. Thus 3 mg. of Ni had been replaced by 11.2 mg. of Ag and 52.6 mg. of Ag were occupying the "free places"; the calculated amount for this last figure would be $98/2 = 49$ mg. Cobalt, in most of the samples, showed hardly any diminution of the value of 18 mg. found in former experiments with Co alone. Theoretically, two-thirds of the places available could be occupied by $\frac{2}{3} \times 98 = 65.3$ mg. of Ag. The average value, actually reached, was around 70 mg. With the Ni and, still more, with the Co experiments, however, the figures showed large fluctuations on account of the disturbing factors mentioned intervening in the case of silver and, perhaps, additional factors, due to the presence of the other metal. Thus, the analogous behavior of silver to copper in these last two types of experiments may be stated only with a certain reserve.

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[1] *Cf.* (1) *Biochem. Z.*, **287**, 86.

SUMMARY

1. There exists a silver compound of casein analogous to the previously described metallic compounds of protein in alkaline medium, especially similar to the copper compound; this Ag compound is disintegrated by acid. The silver oxide, formed simultaneously in the alkaline medium and held in colloidal solution by the protein, is, on addition of acid, precipitated along with the protein. The amount of silver ions then found in the protein filtrate is close to the figure of 98 mg. per 0.5 gm. of casein which corresponds to 58 mg. of copper in previous experiments.
2. The silver compound, however, is less stable than the compounds of copper, nickel, and cobalt; it is disintegrated under the influence of aging of the solution due to standing and by increase of temperature.
3. The silver may be displaced from its positions in the peptide chains by copper, and, to a lesser degree, by Ni and Co. Cu, Ni, and Co are only to a small extent displaced by added silver salt, although in the latter two silver enters the casein in order to occupy the "free places," as does Cu.

The earlier experiments described in this paper were carried out in the Institute for Medical Chemistry at the University of Vienna, with the collaboration of R. Kretschmayer and V. Friedl; while continuing this work at The Johns Hopkins University, I had temporarily the assistance of Miss Virginia White, and, later, of Mr. Albert Reisfeld, to both of whom I wish to extend my thanks.

BIBLIOGRAPHY

1. Lieben F., and Jesserer, H., *Biochem. Z.*, **285**, 36 (1936); **287**, 84 (1936), **292**, 403 (1937); **297**, 369 (1938).
   For a summary see Lieben F., *Nature*, **142**, 463 (1938).
2. Pauli, W., and Matula, J., *Biochem. Z.*, **80**, 187 (1917). Goigner, E., and Pauli, W., *Biochem. Z.*, **235**, 271 (1931).
3. Kruyt, H. R., and Boelman, A. B., *Kolloid.-Beihefte*, **35**, 165 (1931)
4. Carroll, B. H., and Hubbard, D., *Bur. Standards J. Res.*, **7**, 811 (1931).
5. Kolthoff, I. M., and Sandell, E. B., Textbook of quantitative inorganic analysis, New York, 684 (1941).
6. Engle, W. D., and Gustavson, R. G., *Ind. and Eng. Chem.*, **8**, 901 (1916).

THE PHOSPHOLIPASE OF THE VENOM OF THE COTTONMOUTH MOCCASIN (AGKISTRODON PISCIVORUS L)*

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Snake venoms contain an enzyme which partially hydrolyzes phospholipids, liberating fatty acids and monoglyceride derivatives known as lysophospholipids (lysolecithins and lysocephalins). This enzyme has been named lecithinase A (1), or simply lecithinase, but in view of its reactivity towards both lecithins and cephalins (2) phospholipase is preferred as a more general term (3). The enzyme has been crystallized by Slotta and Fraenkel-Conrat (4) from the venom of *Crotalus terrificus*, and has been prepared in impure form from pancreas (3, 5). It is probably present in many other tissues as well (6). In the reaction between phospholipase and phospholipids, only one of the two fatty acids of each substrate molecule is hydrolyzed, and since the early work of Lüdecke (7), who separated the liberated acids as an oil ("oleic acid"), the general opinion has been that such acids are unsaturated. On the other hand, Levene, Rolf, and Simms (2) demonstrated that lysophospholipids derived from egg yolk after incubation with cobra venom contained only saturated acids. Lysophospholipids, like their parent phospholipids, are insoluble in acetone, but unlike the latter are insoluble also in ether. This differential solubility has been used as a method for separating them from unchanged phospholipids. They have a powerful lytic action on many types of cells, although interest has been confined as a rule to their effect on erythrocytes. The lysis of red cells, indeed, has provided the commonest method for detecting the presence of phospholipase, or for judging the extent of formation of lysophospholipids. Some, at least, of the symptoms of snake bite are due to lysophospholipid formation in the body. The subject has been reviewed by Belfanti *et al.* (8) and by Ercoli (9).

In the present communication, an attempt has been made to provide methods for a quantitative description of the action of phospholipase on phospholipids during the initial stages of the reaction, since such methods are required in the study of enzyme purification, activation, and inhibition.

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For this purpose a readily available venom, that of the cottonmouth moccasin (*Agkistrodon piscivorus* L), was selected, with the petroleum ether-soluble phospholipids of beef brain acting as substrate. Experience showed that neither of the two methods mentioned previously was satisfactory. It was found possible, however, to follow the course of the reaction by isolation and micro titration of the liberated fatty acids, even when these constituted as little as 1 to 2 per cent of the reaction mixture. This procedure, although less simple than could be desired, was more nearly quantitative than was expected, and this, together with a reasonable rapidity in execution, led to its adoption. In addition, conditions were found for the production of a zero order reaction between enzyme and substrate, although, owing to our imperfect knowledge regarding the structure of the phospholipids, to the impurity of the substrate used, and to the mixed nature of the liberated fatty acids, absolute values for reaction constants could not be obtained. Ratios of reaction constants, however, which provide an adequate technique for investigation of the reaction, were readily obtained by the zero order method.

EXPERIMENTAL

Enzyme—The source of phospholipase for all experiments was the venom of the cottonmouth moccasin (*Agkistrodon piscivorus* L). This venom is reactive (10), relatively inexpensive, and easy to obtain in dried form.[1] Since it appeared to be inhibited easily, all glassware, after preliminary cleaning, was boiled briefly in dilute nitric acid and then thoroughly rinsed. Redistilled water was used in the preparation of all reaction mixtures.

Substrate—The routine use of highly purified phospholipids was considered to be inadvisable, for these are difficult and tedious to prepare, and are still imperfectly characterized. For the purposes of this investigation, therefore, the petroleum ether-soluble fraction of beef brain phospholipids was used. This fraction was prepared as follows:

5 pounds (2.3 kilos) of fresh beef brain were finely divided in a blending machine in the presence of an equal volume of acetone. The mixture was diluted with 3 liters of acetone, shaken, filtered, and the residue reextracted with acetone for 6 hours. It was then extracted once with alcohol, and twice with ether, over a period of 24 hours, at room temperature. The alcohol- and ether-soluble material, after removal of the solvents *in vacuo*, was suspended in 400 ml. of chloroform, and added dropwise to 2 liters of acetone. The precipitate which formed was separated from the supernatant solution by decantation. A second, and sometimes a third, precipitation was made, after which the acetone-insoluble fraction was sus-

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pended in petroleum ether, and stored at 3° for 24 hours. The voluminous white precipitate which appeared (mainly cerebrosides and sphingomyelins) was separated by centrifugation and discarded; and the supernatant solution, after making to volume with petroleum ether, was stored at 3° until needed.

The mixture obtained in this manner weighed about 70 gm. In seven different preparations the molecular ratios of choline (11) to phosphorus (12) varied from 0.20 to 0.27 (3.0 to 3.3 per cent phosphorus by weight), indicating the presence of both lecithins and cephalins, with the latter predominating. Fatty acids, determined gravimetrically after saponification, comprised 60 to 65 per cent by weight of the substrate. Iodine numbers (13) of these acids varied from 106 to 115. Acetal phospholipids[2] made up 17 to 20 per cent, and cholesterol 3 to 4 per cent of the mixture (15). Small amounts of sphingomyelins, cerebrosides, water, and other extraneous substances were undoubtedly present, although no analyses were made. For purposes of calculation, however, these were assumed to comprise 5 per cent of the mixture. In some instances the substrate was stored as much as 6 weeks before being completely utilized. During this time it darkened considerably in color, and developed a small blank titration (*vide infra*). Its reactivity with phospholipase, however, decreased only slightly.

No attempt was made to obtain extensive analytical data on the nature of the lecithins and cephalins in the obviously impure substrate, since primary emphasis in this investigation concerned the development of methods applicable to subsequent studies on the enzyme and on specific substrates. As a guide to the relative proportions of lecithins and cephalins in crude mixtures of the type used here, experience in this laboratory has been that molecular ratios of choline to phosphorus are reasonably reliable. This opinion was weakened, with respect to brain phospholipids, by the appearance (after this investigation was concluded) of a report by Chargaff, Ziff, and Rittenberg (16), in which it was shown that only 50 per cent of the non-amino nitrogen in brain lecithin and cephalin mixtures could be identified as choline. As a result, the choline to phosphorus ratios given here can be regarded strictly only as ratios of choline to non-choline phospholipids, and much more loosely as ratios of lecithins to cephalins.

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[2] Acetal phospholipids (plasmalogens) were determined by hydrolysis and isolation of the aldehydes (plasmals) For example, 7.8 gm of the mixed phospholipids were emulsified in 200 ml of water, and made 0 1 N with respect to hydrochloric acid. After standing 1 hour at room temperature, the emulsion was extracted repeatedly with petroleum ether, and the combined extracts reduced to a small volume. Addition of excess acetone and a few drops of alcoholic magnesium chloride precipitated the phospholipids, and the aldehydes, which remained in solution, were dried and weighed. The yield was 0.865 gm , *i.e.*, 11 per cent of the initial weight of material, or 20 per cent if converted to terms of the acetal phospholipids originally present. The product was semisolid, light yellow in color, and gave an intense Feulgen aldehyde reaction (14) It was completely soluble in petroleum ether after drying, and was insoluble in dilute alkali.

The important reports of Folch and Schneider (17) and of Folch (18) on the multiple nature of the cephalins also appeared after this work was initiated. The method used by these authors for preparing crude brain phospholipids was similar to the method described here, and it is probable, therefore, that serine, ethanolamine, and inositol cephalins were present in the substrate.

Reaction Mixture—The required amount of substrate was removed from the stock petroleum ether solution, precipitated with acetone, dried *in vacuo*, and weighed. It was then ground in a mortar with a little phosphate buffer solution (0.05 M, pH 7.0), and diluted with buffer to the desired concentration (usually 3 per cent). Emulsions prepared in this way were quite stable, and showed no tendency to liberate acids when incubated in the absence of enzyme. A solution of venom was added to the emulsion (in most experiments, 10 mg. of venom per gm. of substrate) and the mixture was incubated at 37°. When it was desired to stop the reaction before its completion, 3 volumes of alcohol, or enough 0.2 M basic lead acetate to make the final concentration 0.01 M, were added. The liberated fatty acids were then isolated and titrated.

Determination and Properties of Fatty Acids—The reaction mixture, diluted with alcohol, was transferred to an evaporating dish and dried on a steam bath, with addition of alcohol in the terminal stages of the evaporation A 10 ml aliquot of the reaction mixture could be dried completely in half an hour. The residue was then extracted thoroughly with small amounts of chloroform, the extracts combined in a 40 ml. conical centrifuge tube, and reduced to 3 to 4 ml. by boiling. 20 ml. of acetone were poured into the cool solution, and saturated alcoholic magnesium chloride was added dropwise until the precipitate of phospholipids and lysophospholipids was well formed, and the supernatant solution was translucent or clear. After centrifugation, the supernatant solution, which was sometimes slightly turbid, was transferred by decantation to a second, similar tube, and evaporated. During this evaporation the acetone-insoluble residue in Tube 1 was suspended in 2 ml. of petroleum ether, and reprecipitated with 10 ml. of acetone. The supernatant solution, after centrifugation, was added to the acetone-soluble residue in Tube 2, and evaporated to dryness. The residue consisted of fatty acids, together with small amounts of phospholipids, cholesterol, and magnesium chloride. Traces of phospholipids were completely removed by dissolving the mixture in petroleum ether, and precipitating with acetone and 1 or 2 drops of magnesium chloride solution. When centrifuged, the clear solution was decanted into Tube 3, and evaporated. Solution of the residue in 15 ml. of petroleum ether, and washing with 10 ml. of distilled water, freed the fatty acids of traces of magnesium chloride. Washing was effected with a small, motor-driven glass stirrer. The supernatant petroleum ether solution, after centrifuging, was aspirated into a 50 ml. Erlenmeyer flask and evaporated to dryness.

Acids isolated in this manner were liquid at room temperature, and varied in color from a very pale yellow to dark brown, depending upon the age of the phospholipid preparation from which they were derived. When dried *in vacuo*, a small, white, crystalline precipitate usually separated from them. This was identified as cholesterol, and appeared to be the single contaminating substance. If the acids were to be expressed in terms of standard base, they were dissolved immediately in 10 ml. of neutral alcohol, the solution was heated to the boiling point, and titrated with 0.04 N sodium hydroxide solution, phenolphthalein being used as an indicator. The burette used was graduated to 0.01 ml. When the acids obtained weighed more than 40 mg., they were made to volume, and aliquots were taken for the titration. The use of more than 4 ml. of standard base is inadvisable, since at this point a turbidity appears which obscures the end-point of the titration.

When it was desired to determine the weight, mean molecular weight, or iodine number of the fatty acids, the cholesterol had first to be removed. This was accomplished by extracting a petroleum ether solution of the mixture with dilute alkali, and discarding the residual petroleum ether solution. The aqueous soap solution was then acidified, and reextracted with petroleum ether. After washing with water, this extract was evaporated, the residue of acids dried *in vacuo*, and weighed. Mean molecular weights (mean neutral equivalents) were determined by titration; iodine numbers were obtained by the micromethod of Yasuda (13).

Success in the isolation and micro titration of fatty acids by the method described depends in large measure upon the absence of all other acids or bases from the final alcoholic solution. Both phospholipids (cephalins) and magnesium chloride are acidic, and must be completely removed. Glassware was cleaned in the usual manner, and, after rinsing, was refluxed for a few seconds with alcohol vapor.

A negative analysis for phosphorus demonstrated the absence of phospholipids from the final alcoholic solution. 650 mg. of freshly prepared brain phospholipid, when subjected to the procedure, gave a titration of 0.05 ml., compared with 0.04 ml. for the alcohol alone. As the stock petroleum ether solution of phospholipids aged, it acquired a blank titration of as much as 0.15 ml., which necessitated a small correction in the experimental results. Separation of fatty acids from phospholipids was shown to be reasonably quantitative, even in the presence of a large excess of the latter. For instance, 5.4 mg. of unsaturated acids, after admixture with 700 mg. of phospholipids, were recovered with a loss of 10 per cent, and 10 mg. were recovered with an error not exceeding 5 per cent. 10 mg.

of acids (mean molecular weight, 300) represent 0.83 ml. of 0.04 N sodium hydroxide.

As previously stated, acids obtained by enzymic hydrolysis of fresh preparations of substrate were almost colorless oils at room temperature. Mean molecular weights ranged from 295 to 308; iodine numbers from 195 to 222. The Yasuda method for determination of iodine numbers has been shown to give 90 per cent of the theoretical values for the more highly unsaturated compounds, *e.g.*, methyl linoleate (19), and values found by this method to exceed 150 were corrected accordingly. The macromethod of Hoffman and Green (20) gave theoretical values for methyl linoleate, but was not suited to the small amounts of acids usually available.

Although it has been generally believed that phospholipase hydrolyzes only unsaturated acids from the phospholipids, the literature revealed no definite proof. This belief was confirmed by a microfractionation into liquid and solid acids (21), with 45.8 mg. of acids obtained from a fresh preparation of substrate in 69 per cent yield (*vide infra*). Solid acids obtained by fractionation weighed 2.7 mg. (5.9 per cent), iodine number 112, indicating that they were, for the most part, not saturated acids, but solid isomers of the unsaturated acids.

A selective action of phospholipase towards acids of varying degree of unsaturation, or of different molecular weights, appeared possible. This was tested experimentally by determining the iodine number and mean molecular weights of the acids in their progressive liberation during the initial stages of the reaction. Although the last aliquot withdrawn from the reaction mixture contained 5 times as much acids as the first, no change in iodine number or mean molecular weights was observed. Iodine numbers varied at random from 198 to 208; mean molecular weights from 294 to 305. This result, with respect to iodine numbers, confirmed that of Chargaff and Cohen (10), who showed that the iodine number of the fatty acids from brain lecithins did not change when these were partially converted to lysolecithins.

Reaction of Phospholipase with Substrate Constituents—There has been general agreement that phospholipase reacts with both lecithins and cephalins of emulsified egg yolk (2, 10, 22), although Chargaff and Cohen (10) detected no action on purified brain cephalins. The enzyme (from bee venom) has been reported to attack sphingomyelins also (23). In the present investigation, although no detailed study of the action of phospholipase on purified phospholipids was made, it was desirable to determine the reactivity of the *venom* towards the constituents of the impure substrate.

Cerebrosides and Sphingomyelins, Acetal Phospholipids, and Lysophospholipids—None of these substances reacted.

Cerebrosides and sphingomyelins were obtained in crude form as the acetone-insoluble, petroleum ether-insoluble lipid fraction of beef brain.

Failure of the acetal phospholipids to react was determined indirectly. Each of two samples of brain phospholipids weighing 0.72 gm. was incubated for 22 hours, the one with venom added, the other without venom. Each sample was then subjected to the procedure for the isolation of liberated acids, which would also serve to remove liberated aldehydes. The acetone-insoluble residues were then emulsified in 0.1 N hydrochloric acid, and the hydrolyzed aldehydes were isolated by a method already described. In the venom-incubated sample, the yield of aldehydes was 8.4 per cent of the weight of the original sample; in the control sample, the yield was 9.0 per cent. Repetition of the experiment showed a smaller difference between samples.

Evidence that venom did not decompose phospholipids beyond the lysophospholipid stage was furnished by its non-reactivity towards lysophospholipids isolated (*a*) by solvent fractionation from enzymically decomposed egg yolk phospholipids and (*b*) by the cadmium chloride procedure of King and Dolan (22).

Lecithins and Cephalins—The results of several experiments lent support to the belief that both these substances were reactive. Evidence for this was furnished, for example, by experiments in which the reaction was carried as far as possible towards completion. In one experiment, 1.5 gm. of substrate (as a 3 per cent emulsion) was incubated with 15 mg. of venom for 24 hours. The acetone-insoluble residue, after separation of the liberated fatty acids, was emulsified, reincubated with venom, and additional acids isolated. This procedure was repeated twice more, the fourth incubation yielding virtually no free acids. The combined weight of acids was 386 mg.; mean molecular weight, 298; iodine number 178.[3] Lysophospholipids isolated from the reaction mixture (*vide infra*) had essentially the same choline to phosphorus ratio (0.20) as the substrate (0.24). In order to calculate the extent of the reaction, and in view of the substrate composition as previously detailed, lecithins and cephalins were assumed to comprise 75 per cent by weight of the original mixture, with the remaining 25 per cent consisting primarily of non-reactive cholesterol, cerebrosides and sphingomyelins, and acetal phospholipids. On the basis of mean molecular weights for the fatty acids and for the mixed lecithins and cephalins of 300 and 800, respectively, the yield of acids was then found to be 92 per cent theoretical. From this high value, taken in conjunction with the observed ratios of choline to phosphorus, there could be no serious doubt that lecithins and cephalins were reactive.

This conclusion was supported by other experiments in which the reaction was made as complete as possible during a single incubation. For example, 1.88 gm. of substrate were incubated for 11 hours with 40 mg. of venom. This excessive quantity of enzyme was then supplemented by an additional 30 mg. and incubation continued another 4 hours. The yield of acids was 0.382 gm.; mean molecular weight 308; iodine number 211; calculated yield of acids, 72 per cent theoretical. As in the preceding experiment, lysophospholipids isolated from the reaction mixture contained choline and phosphorus in a ratio comparable to that of the substrate (0.22).

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[3] Iodine numbers of acids isolated in experiments of this kind were always low, due to oxidation during the repeated incubation of the substrate.

A quantitative separation of lysophospholipids from the reaction mixture, by means of their insolubility in ether, was not attained. Thus, an experiment which yielded fatty acids calculated by the foregoing method to be 79 per cent theoretical gave ether-insoluble matter calculated (as lysophospholipids) to be 19 per cent theoretical. The following results were obtained on analysis: phosphorus 5.90 per cent (substrate, 3.10 per cent), choline to phosphorus 0.22 (substrate, 0.27), mixed fatty acids 31.6 per cent, iodine number 30 (substrate, 62.7 per cent, iodine number 115). In experiments similar to this, but in which purified lecithins and cephalins were employed as substrates, Chargaff and Cohen (10) reported that lysolecithins could be isolated in yields of 15 to 50 per cent by weight of the initial substrate, but that cephalins were non-reactive. Since isolation of fatty acids was not made by these workers, complete correlation of their results with our own is not possible at the present time.

Kinetics—Although a detailed kinetic investigation is seldom, if ever, justified when, as in the present instance, both enzyme and substrate are impure, an accurate study of enzyme purification, activation, or inhibition requires a method for evaluation of reaction constants, or of ratios of reaction constants, during the initial stages of the reaction. For the direct determination of reaction constants, enzyme-catalyzed hydrolytic reactions can sometimes be made to simulate first order reactions by suitable reduction in the initial substrate concentration. This was not feasible in the case of phospholipase, due to the concomitant increase in volumes of substrate emulsion required for analysis. As a result, two methods for determining ratios of reaction constants were examined.

Reciprocal Time Method—In a reaction of a given type, the ratio of the reaction constants for a given condition and its variant equals the ratio of the reciprocals of the times required to effect a given change (24). This method was found to be reasonably satisfactory. Although details will not be given here, it was shown that the rate of reaction was directly proportional to the enzyme concentration. The method was used also to determine the stability of venom in buffer solution. A 0.1 per cent solution was heated for 30 minutes at varying temperatures, after which its reactivity was determined. It was found that the enzyme was not inactivated at 35°, but underwent 72, 86, 96, and 99 per cent inactivation at 53°, 60°, 75°, and 90°, respectively.

Zero Order Reaction—Here the rate is a linear function of time, and independent of substrate concentration. The ratio of reaction constants, therefore, is determined directly by finding the ratio of the amounts of substrate changed in a given time; and when, as in the case of phospholipase, the rate of reaction is directly proportional to the enzyme concentration, the latter ratio is equal also to the ratio of the amounts of active enzyme. In this way the study of the enzyme is placed upon a simple basis.

The reaction between phospholipase and substrate was zero order during its initial stages when the substrate concentration was increased to 6.5 per cent or more, and the reaction conducted at 22°. In Fig. 1 is shown the effect of increased concentration of substrate on the reaction velocity at 22° and 37°. Aliquots of varying volumes were transferred from a 9 per cent substrate emulsion into glass-stoppered Erlenmeyer flasks. Each aliquot was then diluted to 19 ml. with buffer, mixed with 1 ml. of venom solution, and allowed to react for 10 minutes. At 22° the rate of reaction was nearly constant at substrate concentrations greater than 6 per cent; whereas at 37° the rate increased steadily over the range of concentrations examined. A substrate decomposition curve under the conditions applying at 22° is shown in Fig. 2. In this experiment the initial substrate concentration was 6.5 per cent. The curve obtained was linear (zero order) over a range represented by 1 to 3 ml. of standard base.

In Fig. 2 the linear part of the curve, when extended, does not meet the origin, but a point considerably above this, at about 0.8 ml. of standard base. This could be due (*a*) to an excessively large blank titration or (*b*) to incomplete inhibition of the reaction by basic lead acetate at the time of removal of aliquots for analysis. Both of these possibilities were examined, with negative results It may be, therefore, that in this mixed substrate there was present a small quantity of material which reacted with the enzyme at a rate much greater than did the bulk of the substrate. Although this question was not examined further, the usefulness of the method was not impaired, provided that the curves obtained under different conditions extended to the same point. That this is actually the case (within experimental error) was shown in an experiment (Fig. 3) in which three different substrate preparations reacted with the enzyme, (*a*) the stock petroleum ether-soluble substrate, and (*b*) and (*c*) alcohol-soluble and insoluble substrates prepared from (*a*). Choline to phosphorus ratios of (*a*) and (*b*) were 0.24 and 0.57 respectively, with (*c*) being choline-free.[4]

Application of the method was made in determining the heat stability of phospholipase at 47°. For this purpose a 0.23 per cent solution of venom was maintained at 47° for periods up to 3 hours, before being added to a

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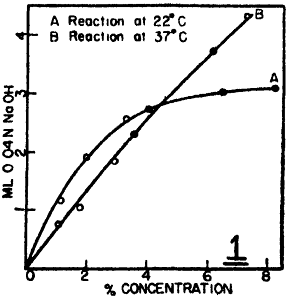
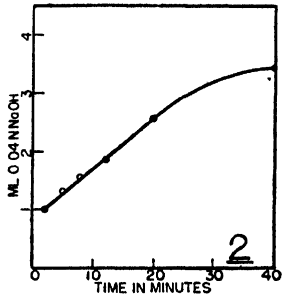
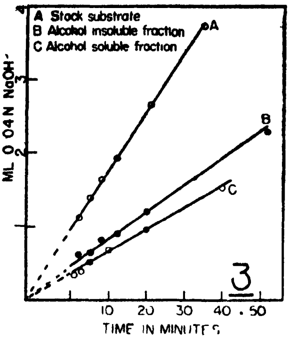
[4] The curious failure of two fractions of the stock substrate to react at as great a rate as the stock substrate itself was confirmed in another experiment. Possible explanations are numerous, but would be premature at the present time. The results have been included (1) for reasons given in the text and (2) because they serve to illustrate the usefulness of the method as applied to this type of problem.







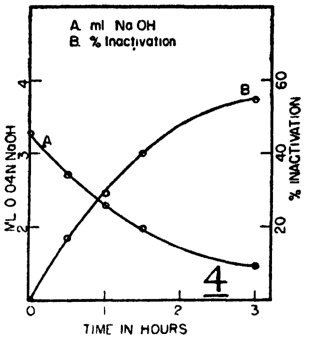


FIG. 1. The effect of substrate concentration on the rate of reaction. Titration values represent the acids liberated in 10 minutes from 20 ml. of substrate emulsion of varying per cent concentrations. Curve A, 0.25 mg. of venom per ml of emulsion, temperature 22°; Curve B, 0 16 mg of venom per ml of emulsion, temperature 37°. In Curve B the decreased enzyme concentration accounts for the decreased initial slope.

FIG. 2. Hydrolysis of phospholipids by phospholipase as a zero order reaction. Titration values represent the acids liberated from 10 ml. aliquots of a 6.5 per cent substrate emulsion. Concentration of venom, 0.25 mg. per ml. of emulsion; temperature 22°. The curve is linear between approximately 1 to 3 ml. of standard base.

FIG. 3. Showing the extrapolation of zero order curves to a common origin. Titration values represent the acids liberated from 10 ml. aliquots of 7.5 per cent substrate emulsions. 0.25 mg. of venom per ml. of emulsion; temperature 22°.

FIG. 4. Illustration of the zero order reaction method, applied to the heat inactivation of phospholipase at 47°. Curve A, titration values of acids liberated in 15 minutes from a 7.5 per cent substrate emulsion, plotted against time of preheating of a 0.23 per cent solution of venom. Concentration of venom at zero time of heating, 0.25 mg. per ml. of emulsion Curve B, per cent inactivation of enzyme, derived from Curve A (see the text).

7.5 per cent substrate emulsion at 22°. After 15 minutes of incubation, *i.e.* for a period of time during which the reaction was zero order (Fig. 3), the reaction was stopped, and the liberated acids titrated (Fig. 4). In

Fig. 4 are plotted also the results obtained when all titration figures were expessed in per cent decrease from the value at zero time of heating at 47°. As stated previously, these percentages are then equal to the per cent inactivation of the enzyme by heating.

DISCUSSION

Considerable care is required in order to isolate and titrate acids according to the method described, and with the precision claimed. On the other hand, the method, when mastered, enables the operator to carry out six to ten analyses in a period of 3 hours, and this is probably sufficiently rapid for application, for example, to studies involving the separation and purification of phospholipase from animal tissues. In some of the latter experiments the substrate, previously stored in petroleum ether solution, was stored at 3° as a 7.5 per cent emulsion (in phosphate buffer, pH 7.0), a procedure which was entirely satisfactory and which eliminated the necessity for preparing a fresh emulsion for each set of analyses.

There are two definite advantages, apart from kinetic considerations, in the adoption of a zero order reaction technique. These are (1) elimination of the necessity for preparing an emulsion of accurately known concentration, since rate of reaction under these conditions is independent of substrate concentration; and (2) the smaller (though more concentrated) volumes of reaction mixture required for analysis. The adoption for routine use of a 7.5 per cent substrate emulsion would appear to be suitable, since at this concentration the reaction is zero order within fairly wide limits (Fig. 3). More concentrated emulsions are very viscous, making the removal of aliquots inaccurate.

Unfortunately the work reported here was interrupted before extensive application of the methods described was possible. On this account, for example, the reaction of phospholipase with both lecithins and cephalins of the substrate, made probable by experiments described in the text, cannot be regarded as proved, particularly in view of the failure of Chargaff and Cohen (10) to obtain any reaction with purified brain cephalin. From the fact that these authors, and others (2, 22), have found cephalins in egg yolk emulsions to be reactive, however, it would appear that the process of purification may lead to non-reactivity. In this connection it may be added that little is known regarding substances which inactivate phospholipase. The heat stability of the enzyme is considerably less than has been reported for pancreatic phospholipase (3) and for cobra venom (25), but this discrepancy is to be expected in view of the fact that the latter results were not obtained by determination of reaction rates.

SUMMARY

1. A method is described for following the partial hydrolysis of phospholipids by the phospholipase of moccasin venom. 10 mg. of fatty acids,

mixed with 700 mg. of phospholipid, may be isolated and titrated with less than 5 per cent error.

2. Application of this method was made in studying the initial stages of the reaction. Conditions were found for the production of a zero order reaction, and the usefulness of this type of reaction in the experimental study of the enzyme is described.

3. Evidence is presented to show the reactivity of both lecithins and cephalins in the substrate. Liberated acids were unsaturated, and there was no preference on the part of the enzyme for acids of varying degrees of unsaturation, or of varying molecular weight. The reaction was calculated to approach completion when reaction products were removed.

4. Cerebrosides, sphingomyelins, acetal phospholipids, and lysophospholipids did not react with venom.

The author wishes to acknowledge his great indebtedness to Dr. W. R. Bloor and Dr. R. G. Sinclair, who made this work possible.

BIBLIOGRAPHY

1. Contardi, A , and Ercoli, A , *Biochem. Z.*, **261**, 275 (1933).
2. Levene, P. A., Rolf, I P , and Simms, H. S , *J Biol Chem.*, **58**, 859 (1923–24).
3. Ogawa, K., *J. Biochem.*, Japan, **24**, 389 (1936).
4. Slotta, K. H., and Fraenkel-Conrat, H L , *Ber chem Ges* , **71 B**, 1076 (1938).
5. Gronchi, V., *Sperimentale*, **90**, 223 (1936)
6. Francioli, M , *Fermentforschung*, **14**, 241 (1934).
7. Lüdecke, K., Dissertation, Munich (1905)
8. Belfanti, S., Contardi, A., and Ercoli, A , in Nord, F F., and Weidenhagen, R., Ergebnisse der Enzymforschung, Leipzig, **5**, 213 (1936).
9. Ercoli, A., in Nord, F F , and Weidenhagen, R., Handbuch der Enzymologie, **1**, 480, Leipzig (1940)
10. Chargaff, E., and Cohen, S S , *J. Biol. Chem* , **129**, 619 (1939).
11. Beattie, F. J R., *Biochem J* , **30**, 1554 (1936).
12. Man, E. B., and Peters, J. P , *J. Biol. Chem.*, **101**, 685 (1933).
13. Yasuda, M., *J. Biol. Chem.*, **94**, 401 (1931–32).
14. Feulgen, R., and Voit, K , *Arch. ges. Physiol.*, **206**, 389 (1924)
15. Kelsey, F. E., *J. Biol. Chem.*, **127**, 15 (1939).
16. Chargaff, E , Ziff, M., and Rittenberg, D , *J Biol Chem.*, **144**, 343 (1942).
17. Folch, J., and Schneider, H. A., *J. Biol. Chem* , **137**, 51 (1941).
18. Folch, J., *J. Biol. Chem* , **139**, 973 (1941); **146**, 35 (1942).
19. Riley, R. F., Thesis, University of Rochester (1942).
20. Hoffman, H. D., and Green, C. E , *Oil and Soap*, **16**, 236 (1939).
21 Sinclair, R. G., *J. Biol. Chem* , **111**, 261 (1935).
22. King, E. J , and Dolan, M , *Biochem. J.*, **27**, 403 (1933).
23. Magistris, H., *Biochem. Z* , **210**, 85 (1929).
24. Bodansky, O., *J Biol. Chem* , **120**, 555 (1937).
25. Rousseau, E., *Compt rend Soc. biol* , **117**, 565 (1934).

FREE FATTY ACIDS IN ANIMAL TISSUES*

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From time to time, the presence in various animal tissues of considerable amounts of the higher molecular weight fatty acids in the "free" state has been reported (1–5). Among the tissues in which these free acids were found are blood (1, 4), liver (2, 3), heart, muscle, lung, and kidney (1, 2), and intestinal mucosa (5). As an example, Kelsey and Longenecker (4) found that of the total fatty acid composition of the acetone-soluble beef plasma lipids, 24.1 per cent occurred as free fatty acids. It has been generally recognized that such acids do not necessarily occur free in living tissues, and that their presence may be due to the autolytic decomposition of cell lipids during the preparation of extracts for analysis. Apparently this possibility has not been adequately tested. Experiments conducted in this laboratory showed that autolysis of liver phospholipids proceeds rapidly upon removal of the organ from the body, and suggested that these and the glycerides might be responsible for the free acids so often found. Accordingly, experiments were carried out which indicated that the free fatty acid content of whole mice, and of cat liver, is very small, and that autolysis of cell lipids is probably the source of most of the acids ordinarily found.

EXPERIMENTAL

Autolysis of Liver Phospholipids—Preliminary experiments on the autolysis of liver phospholipids were performed as follows. Portions of the livers of adult rats or cats were removed, and transferred quickly to weighed conical centrifuge tubes containing about 20 ml. of alcohol and a little sand. The tissue was ground immediately and the tubes and contents reweighed. Other portions of the liver were then removed, weighed, and (*a*) allowed to autolyze at room temperature, (*b*) ground and autolyzed at room temperature, or (*c*) ground, suspended in phosphate buffer, pH 7.2, and autolyzed at 37°. Phospholipids were isolated from control and autolyzed samples by the usual methods, and the amounts of fatty acids, phosphorus (6), and choline (7) were determined and compared. Fatty acids were determined gravimetrically after saponification of the phospholipids. The results of experiments with unground liver, autolyzed for a few minutes at room temperature, always showed a decrease of about 8 per cent in phospholipid fatty acids, phosphorus, and choline when compared with the control values. When the liver was ground and allowed to autolyze, hydrolysis of the phospholipids increased to about 15 per cent, and then came to an abrupt stop. On the other hand, when the ground liver was buffered and then autolyzed, hydrolysis went much further. One of several experiments of this type is described below.

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* Taken from a thesis submitted by the author to the Graduate School, University of Rochester, 1942, in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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Two adult male rats were anesthetized with nembutal, and their livers were exposed. A portion of about 0.5 gm. was removed from each liver, the two portions were combined, ground in alcohol, and weighed. The remainder of the two livers was combined, weighed, and ground finely with sand. It was then transferred to phosphate buffer at 37° (100 ml. of 0.05 M buffer, pH 7.2, per gm. of tissue), and shaken for 1 minute. 200 ml. aliquots were removed at intervals up to 12 hours. Each aliquot as withdrawn was transferred to a centrifuge tube, after which the phospholipids and proteins were precipitated with colloidal iron and magnesium sulfate (8) and centrifuged. The supernatant solution was free of phospholipids. Phospholipids were extracted from the precipitate, and analyzed for phosphorus, choline, and fatty acids.

The results of this experiment are shown in Fig. 1, in which per cent hydrolysis of phosphorus, choline, and fatty acids, calculated from control and autolyzed samples, is plotted against time. It is apparent that Curves A, B, and C, representing phosphorus, fatty acids, and choline, respectively, parallel one another closely, and approach a maximum hydrolysis of 40 to 45 per cent after 4 hours incubation. Thus a given phospholipid molecule, if hydrolyzed at all, is hydrolyzed rather completely, lecithins and cephalins being degraded at about the same rate. Iodine numbers of the phospholipid fatty acids isolated from control and autolyzed samples varied from 129 to 133.

Free Fatty Acids in Normal and Autolyzed Tissues—The experiments which have just been described showed that free fatty acids ordinarily found in lipid extracts could be due to autolytic hydrolysis of the tissue lipids during the preparation of these extracts. This possibility was tested experimentally. Normal values for free fatty acids, and apparent values following autolysis, were determined on whole mice (less the gastrointestinal tract) and on cat liver. In the determination of normal values, a fast freezing technique was used, since autolysis could be effectively suppressed in this way. Preparation of the lipid extracts, removal of interfering substances, and titration of the acetone-soluble fatty acids were carried out by methods previously described (9).

Experiments with Mice—Thirty young adult male mice, reared on a uniform stock diet, were divided into two equal groups. The animals in Group 1 were used to determine the normally occurring free acids, and those in Group 2 were used to determine the free acids after the tissues were ground in air at room temperature. Details of the experiment are summarized in Table I. Inspection of this table reveals the fact that the amounts of free fatty acids in normal mouse tissue (Group 1) were very small, averaging 2.2 mg. per gm. of dry, fat-free tissue, or 0.55 per cent of the acetone-soluble lipids. On the other hand, the free fatty acids of autolyzed mouse tissues averaged 14.8 mg. per gm. of dry, fat-free tissue, or 4.1 per cent of the acetone-soluble lipids. This is a 7-fold increase over the average normal value.

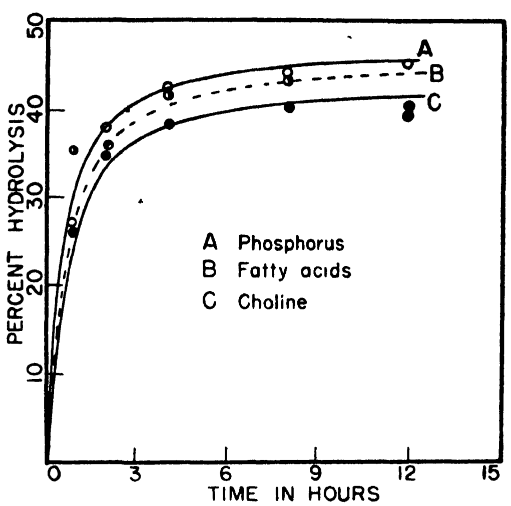

FIG. 1. Autolysis of liver phospholipids in buffered suspension. Curves A, B, and C show the progressive decrease in phospholipid phosphorus, fatty acids, and choline, respectively.

Experiments with Cat Liver—Liver tissue is known to autolyze rapidly, and was therefore chosen for these experiments. The following method was used.

The liver was removed from an anesthetized cat, and frozen as rapidly as possible by laying against the walls of a mortar which had been thoroughly chilled in a freezing mixture of acetone-carbon dioxide ice. 3 or 4 minutes were required for complete freezing. The tissue was then ground in a cold meat chopper, and while still frozen was transferred to 125 ml. Erlenmeyer flasks in lots of 10 to 15 gm. each. These were attached to a high vacuum system, which removed the water from the frozen tissue (10). After 24 hours evacuation, the flasks were sealed under a vacuum and stored at 3° until used. The amount of water removed from the liver by this method was comparable to that removed by desiccation at 100° over phosphorus pentoxide. Total lipids of 0.5 gm. samples of this liver preparation were extracted, and the free fatty acids determined. In addition, 6 gm.

TABLE I

Free Fatty Acid Content of Normal and Autolyzed Mouse Tissue

Group No		Dry fat-free residue	Per cent of acetone-soluble lipids
		mg. per gm	
1 (fifteen mice)	Average	2.2	0.55
	" deviation	0.5	0.14
2 (fifteen mice)	"	14.8	4.1
	" deviation	2.0	1.0

Free fatty acids are expressed as oleic acid.

Group 1 mice served as controls. The anesthetized animals, after removal of the gastrointestinal tract, were immersed in a freezing mixture of alcohol-carbon dioxide ice. Each frozen carcass was then ground in a cold meat chopper and transferred immediately to boiling alcohol Total lipid extracts were prepared, and free fatty acids determined by titration.

In Group 2 animals, the gastrointestinal tract was removed, and the mice were ground, with no preliminary freezing. The minced tissues were allowed to stand for 1 hour at room temperature before the lipids were extracted. In control experiments, it was shown that from a mixture of 5 mg of oleic acid, 100 mg. of phospholipids, and 100 mg. of triglycerides, 90 per cent of the fatty acid could be separated, as measured by titration; or 95 per cent separation could be effected if the initial weight of oleic acid was increased to 10 mg.

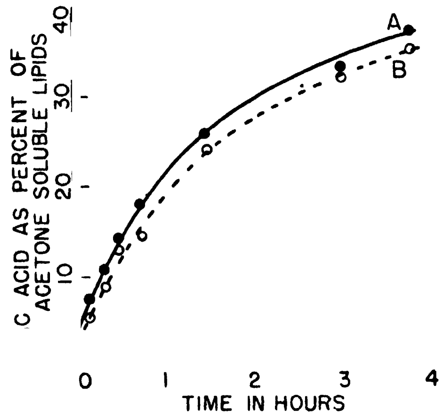

FIG. 2. The progressive increase of free fatty acids in buffered liver suspension. Curve A gives the results of titration of the acetone-soluble lipid fraction. In Curve B the acids were separated from other acetone-soluble lipids, and retitrated.

of the same preparation were pulverized in a mortar, shaken with 400 ml. of phosphate buffer, and incubated at 37°. 50 ml. aliquots were removed from the mixture at time intervals ranging from 10 minutes to 4 hours, and were diluted immediately with 100 ml. of boiling alcohol. Total lipid extracts were then prepared and analyzed for free fatty acids.

Curve A, Fig. 2, shows the results of one of three such experiments, all of which gave similar results. Free fatty acids are expressed as per cent of oleic acid in the acetone-soluble lipids. On this basis, control samples (at zero time) gave values of 2.2 to 2.5, and autolyzed samples gave rapidly increasing values, the per cent after 4 hours incubation being 38. That the acids titrated were actually fatty acids was shown by removing them from other acetone-soluble lipids as the sodium soaps, acidifying, and titrating weighed portions of the precipitated acids with standard base. Curve B, Fig. 2, demonstrates the nearly quantitative isolation of the fatty acids. In all cases a mean molecular weight of about 300 was obtained.

DISCUSSION

Hydrolysis of the constituent phospholipids during autolysis of liver was first observed by Artom (11), who found a 33 per cent decrease in the liver phospholipids of the dog after a 24 hour incubation of an unbuffered mince. After the experiments reported in the present communication had been completed, Fishler *et al.* (12) and Sperry and associates (13) reported the hydrolysis of the phospholipids of liver slices or pieces, as measured by the changes in phospholipid phosphorus. No study of the constituent fatty acids or choline was made. The chief points requiring emphasis in our experiments are (*a*) the rapid initial loss of phospholipids when the liver is removed, and (*b*) the rather complete hydrolysis of a given phospholipid molecule. The first point is of some importance, for the conditions under which some 15 per cent of the phospholipids are hydrolyzed, *i.e.*, grinding the liver in air at room temperature, are the very conditions which are generally used in the preparation of total lipid extracts. This loss of phospholipids was overcome by grinding the liver in alcohol. The second point, although highly interesting, scarcely warrants discussion at the present time. Failure of the autolytic enzymes to distinguish between the lecithins and cephalins, or the various unsaturated acids, could possibly be interpreted as evidence against the theories of "structural" and "metabolic" phospholipids (14), although the dangers involved in reading the results of autolytic experiments into *in vivo* mechanisms are obvious.

Our results show clearly that the free fatty acid content of normal mice (less the gastrointestinal tract) and of cat liver is very low. It is probably fair to conclude that much higher values reported previously for various tissues have been due, in large part, to inadequate control of the appropriate enzymes during the preparation of the lipids for analysis. This assumption is supported in our experiments by the large increase in free fatty acids which occurred even as the result of a very mild autolysis. Although the values for normal tissues are low, they may in actuality be much too high, for it must be noted that freezing of the tissues never occurred instantaneously, and that, as a result, the enzymes involved in the liberation of fatty acids were almost certainly only partially inhibited during the initial experimental procedures. The control values, therefore, should be regarded as *maximum* values for the tissues which were studied.

SUMMARY

1. When rat or cat liver is extirpated, a part of the constituent phospholipids is rapidly hydrolyzed by the intracellular phospholipases. Within a few minutes, this hydrolysis in intact, isolated liver tissue amounts to about 8 per cent. If the liver is ground, hydrolysis increases to about 15 per cent. When the ground tissue is suspended in buffer at pH 7.2, some 40 per cent of its phospholipids is hydrolyzed within 4 hours. Decreases in phospholipid phosphorus, choline, and fatty acids parallel one another closely. There appears to be no preferential hydrolysis of particular fatty acids.

2. The amounts of free fatty acids which occur normally in tissues are very small, averaging 2.2 mg. per gm. of dry, fat-free mouse tissue and 2.3 per cent of the acetone-soluble lipids of cat liver. These values, due to limitations of the experimental methods, may be regarded as maximum. Autolysis, even for short periods of time, greatly increases the amounts of free fatty acids in these tissues.

The author wishes to express his great indebtedness to Dr. W. R. Bloor and Dr. R. G. Sinclair, who made this work possible.

BIBLIOGRAPHY

1. Bloor, W. R., *J. Biol. Chem* , **59**, 543 (1924); **68**, 33 (1926).
2. Bloor, W. R., and Snider, R. H., *J. Biol. Chem.*, **87**, 399 (1930).
3. Hilditch, T., and Shorland, F., *Biochem. J.*, **31**, 1499 (1937).
4. Kelsey, F. E., and Longenecker, H. E., *J. Biol. Chem.*, **139**, 727 (1941)
5. Lovern, J., and Morton, R., *Biochem. J* , **33**, 330, 1734 (1939).
6. Man, E. B., and Peters, J. P., *J. Biol. Chem.*, **101**, 685 (1933).
7. Beattie, F. J. R , *Biochem. J.*, **30**, 1554 (1936).
8. Folch, J., and Van Slyke, D. D , *Proc Soc. Exp. Biol. and Med* , **41**, 514 (1939).
9. Fairbairn, D., **157**, 633 (1945).
10. Flosdorf, E W., and Mudd, S., *J. Immunol.*, **29**, 369 (1935).
11. Artom, C., *Bull. Soc chim. biol.*, **7**, 1099 (1925)
12. Fishler, M. C., Taurog, A., Perlman, I., and Chaikoff, I. L., *J. Biol. Chem.*, **141**, 809 (1941).
13. Sperry, W. M., Brand, F. C., and Copenhaver, W. M., *J. Biol. Chem.*, **144**, 297 (1942).
14. Sinclair, R. G., *J. Biol Chem.*, **111**, 515 (1935).

MICROBIOLOGICAL METHODS FOR THE DETERMINATION OF AMINO ACIDS

I. ASPARTIC ACID AND SERINE

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Knowledge of the growth factor requirements of various lactic acid bacteria, especially *Lactobacillus casei* and *L. arabinosus* 17-5, has led to the development of useful microbiological methods for the quantitative determination of most of the B vitamins. Recently attention has turned to the amino acid requirements of the lactobacilli. The amino acids necessary for growth of *L. casei* and *L. arabinosus* have been determined (1–4) and satisfactory methods have been developed with these two organisms for the assay of leucine (3–5), isoleucine (3), valine (3–6), arginine (6), phenylalanine (4), tryptophane (7), and glutamic acid (8) in purified proteins and to a more limited extent in natural products.

Although *Lactobacillus casei* or *L. arabinosus* responds quantitatively to arginine, isoleucine, leucine, methionine, phenylalanine, threonine, tryptophane, valine, glutamic acid, tyrosine, and cystine (2–4, 6, 9), irregular results have been obtained with serine, aspartic acid, and lysine (9). Also neither organism requires histidine, proline, hydroxyproline, norleucine, glycine, or alanine for growth, presumably because these amino acids are synthesized by the bacteria. It seemed desirable, therefore, to investigate the amino acid requirements of other lactobacilli which might be used for the assay of those amino acids which cannot be satisfactorily measured with *L. casei* or *L. arabinosus*. The amino acids necessary for growth of *L. delbruckii* LD5[1] have been determined (to be published elsewhere) and a method has been developed for the assay of aspartic acid and serine in purified proteins. The latter is described in the present paper.

Procedure

Inoculum—Stab stock cultures of the organism are carried in a medium of the following composition: 1 gm. of glucose, 0.5 gm. of Bacto-peptone, 0.6 gm. of anhydrous sodium acetate, Salts A and B in half the concentration given in Table I, and 1.5 gm. of agar per 100 cc. of medium at pH

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[1] Kindly supplied by Dr. I. C. Gunsalus. It can be obtained from the American Type Culture Collection, Georgetown University School of Medicine, Washington, D. C., where it is listed as No. 9595.

6.8. The cultures are stored in a refrigerator and subcultured each month. Inoculum for the assay is prepared by transferring a small amount of growth from a stab culture to a centrifuge tube containing 8 cc. of the same medium but without agar. After incubation for 16 to 24 hours at 37°, the cells of the liquid culture are centrifuged, washed with water, and suspended in 20 cc. of water. Occassionally, 2 day-old cultures have been used without any harmful effect. Use of more dilute suspensions tends to give "standard curves" which do not reach the usual maximum of 9 to 10 cc. of titratable acid. 1 drop (about 0.05 cc.) of cell suspension is used to inoculate each tube in the assay.

TABLE I

Basal Medium

dl-Leucine	100 mg.	Glucose	10.0 gm.
dl-Isoleucine	100 "	Sodium acetate (anhydrous)	3.0 "
dl-Valine	100 "	Adenine	10 mg.
l(−)-Cystine	100 "	Pantothenic acid	100 γ
dl-Methionine	100 "	Riboflavin	100 "
l(−)-Tryptophane	100 "	Nicotinic acid	100 "
l(−)-Tyrosine	100 "	Pyridoxamine[*]	100 "
dl-Phenylalanine	100 "	Biotin	0.05 "
dl-Glutamic acid	100 "	Folic acid[†]	0.5[‡] "
dl-Threonine	100 "	Salts A	
dl-Alanine	100 "	K_2HPO_4	250 mg.
dl-Aspartic acid[§]	100 "	KH_2PO_4	250 "
l(+)-Lysine	50 "	Salts B	
l(+)-Arginine	100 "	$MgSO_4\cdot 7H_2O$	100 "
l(+)-Histidine	100 "	NaCl	5 "
dl-Serine[‖]	100 "	$FeSO_4\cdot 7H_2O$	5 "
l(−)-Proline	100 "	$MnSO_4\cdot 4H_2O$	5 "
l(−)-Hydroxyproline	100 "	Adjust to pH 6.8	
dl-Norleucine	100 "	Add distilled H_2O to	250 cc.
Glycine	100 "		

[*] We are indebted to Dr. S. A. Harris, Dr. Dorothea Heyl, and Dr. K. Folkers for this compound.

[†] Kindly supplied by Dr. R. J. Williams as a concentrate.

[‡] Equivalent to 0.5 γ of material of "potency 40,000."

[§] Omitted in assaying for aspartic acid.

[‖] Omitted in assaying for serine.

Assay Medium—The composition of the assay medium is shown in Table I. The amount indicated is sufficient for 50 assay tubes since each tube receives 5 cc. of medium. This is enough for determining one amino acid in three different proteins. In preparing medium for larger assays, allowance should be made for twenty tubes for the standard and ten tubes for each unknown. Stock solutions of the amino acids are prepared in distilled water in a concentration of 10 mg. per cc. Only 5 mg. of leucine are added per cc. to avoid crystallization on standing. Glutamic and aspartic acids are prepared in a concentration of 20 mg. per cc. Tyrosine is dissolved in 1 N NaOH and then diluted with water so that a solution of 10 mg. per cc. of 0.1 N NaOH is obtained. Cystine is dissolved in 2 N HCl and diluted with water to a final concentration of 5 mg. per cc. of 0.2 N HCl. It has been found convenient to prepare 500 cc. quantities of each amino acid at one time and to store these in brown, glass-stoppered bottles, under a thin layer of toluene, in the refrigerator. They can be kept in this manner for several months without noticeable deterioration.

The remainder of the ingredients of the medium, with the exception of glucose and sodium acetate, also are prepared as solutions, so that they can be pipetted conveniently. Salts A is made by dissolving 50 gm. each of K_2HPO_4 and KH_2PO_4 in 500 cc. of distilled water. Salts B is made by dissolving, in order, 20 gm. of $MgSO_4 \cdot 7H_2O$, 1 gm. of NaCl, 1 gm. of $FeSO_4 \cdot 7H_2O$, and 1 gm. $MnSO_4 \cdot 4H_2O$ in 500 cc. of distilled water. 1 cc. of concentrated HCl is added to Salts B to prevent precipitation of salts on standing. A stock solution containing 1 mg. of adenine per cc. is prepared by dissolving 870 mg. of adenine sulfate in 500 cc. of water.

The vitamin solutions are prepared individually as follows: riboflavin 25 γ per cc. of 0.02 N acetic acid, calcium pantothenate 27.2 γ and nicotinic acid 100 γ per cc. of H_2O, biotin (free acid) 0.02 γ per cc. of H_2O prepared for an initial solution of the solid in 20 per cent ethanol, folic acid 1.0 γ, and pyridoxamine 10 γ per cc. of H_2O. All of the above solutions are stored in brown bottles under toluene in the refrigerator. The vitamin solution should be renewed once a month; however, fresh solutions of pyridoxamine should be made at shorter intervals, since the degree of its stability in water is not known at present. The remaining solutions can be kept almost indefinitely.

The medium is similar to that described by Hutchings and Peterson (10). It is not improved by addition of $(NH_4)_2SO_4$ or doubling the sodium acetate or by an increase in folic acid. Growth of *Lactobacillus delbrückii* will not occur if any of the listed vitamins are omitted from the medium, indicating that all are essential. In most of the experiments, pyridoxine hydrochloride was used in place of pyridoxamine. When the latter became available (11, 12), it was found to support greater growth of *Lactobacillus delbruckii* with serine but not with aspartic acid (see below) and is, therefore, to be preferred in the basal medium.

Preparation of Samples for Assay—Proteins are hydrolyzed essentially according to the method of McMahan and Snell (6). 300 mg. of protein are placed in a glass ampule of 5 cc. capacity along with 3 cc. of 10 per cent HCl.

If only small amounts of protein are available, 50 mg. or less may be hydrolyzed with 1 cc. of acid. The ampule is sealed in a flame and autoclaved on its side for 10 hours at 15 pounds pressure. After being cooled, the ampule is broken open and the hydrolysate washed into a small beaker with approximately 25 cc. of water. It is then adjusted to pH 6.8 with 5 N NaOH, filtered through paper if an appreciable amount of insoluble material is present, and brought to a volume of 50 cc. with water. It may be stored under toluene in the refrigerator.

Preparation of Standards—The aspartic acid standard solution is made by dissolving 50 mg. of *l*-aspartic acid in 250 cc. of water. This solution contains 200 γ of aspartic acid per cc. The serine standard solution is prepared by dissolving 25 mg. of *dl*-serine in 250 cc. of water to give a concentration of 50 γ of the *l* isomer per cc. The solutions may be stored under toluene in the refrigerator for at least 3 weeks without loss of potency.

Assay Procedure—For the assay of aspartic acid, 5 cc. quantities of the appropriate basal medium (see Table I), free of aspartic acid, are placed in lipless test-tubes (180 × 22 mm.) supported in wire racks. A standard curve is prepared by adding, in duplicate, amounts ranging from 0 to 4 cc. of the standard *l*-aspartic acid solution, equivalent to 0 to 800 γ of aspartic acid (see Fig. 1 for intermediate values), to separate tubes of medium. To another set of tubes containing 5 cc. of aspartic acid-free medium are added in duplicate 1.0, 1.5, 2.0, 2.5, and 3.0 cc. quantities of the appropriate dilution of the protein hydrolysate being assayed. The total volume in all tubes is brought to 10 cc. with water. The tubes are plugged with cotton and sterilized by autoclaving at 15 pounds pressure for only 13 minutes. On autoclaving, the medium becomes light brown in color; dark brown discoloration indicates excessive sterilization and interferes with the subsequent colorimetric titrations. Each tube is inoculated, aseptically, with 1 drop of *Lactobacillus delbrückii* suspension prepared as described above. The racks of inoculated tubes are incubated for 72 hours at 37° to permit development of the lactobacillus to the maximum degree permitted by the available aspartic acid. Variations of 6 to 8 hours in time and 1° or 2° in temperature do not affect the assay significantly.

The procedure for the assay of serine is exactly the same as that for aspartic acid except that serine is omitted from the basal medium used for both standard and samples and the reference curve is prepared from the serine standard solution with the quantities indicated in Fig. 2.

After incubation, the lactic acid formed by *Lactobacillus delbrückii* from the glucose in the medium is titrated directly in each tube with 0.1 N NaOH, with brom-thymol blue as indicator. The cc. of 0.1 N NaOH required to neutralize the tubes of the standard are plotted against micrograms of aspartic acid or serine to give the reference curves shown in Figs.

1 and 2. Similar standard curves are prepared with each assay. Dilutions of the protein hydrolysate under test are chosen to give titration values which fall on the sharply ascending portion of the standard curves and the

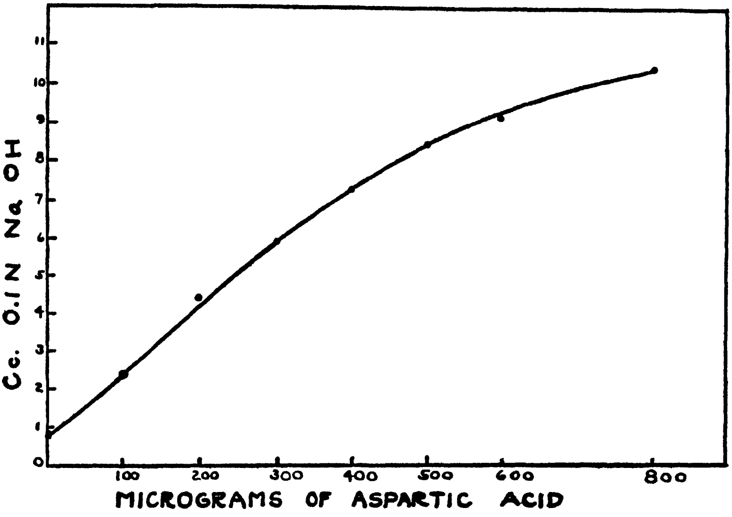

Fig. 1. Standard aspartic acid curve

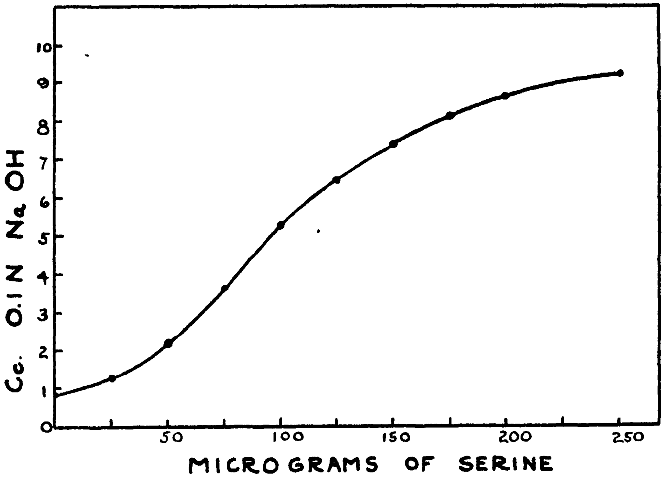

Fig. 2. Standard serine curve

aspartic acid or serine content is read from the appropriate curve. The final value is an average of the figures obtained at the various levels which agree closely (Table II).

Activity of Optical Isomers—The *d* and *l* isomers of aspartic acid are essentially equally available to *Lactobacillus delbrückii*. This is the first recorded exception to previous indications that only the naturally occurring isomer of amino acids can be utilized by lactobacilli (13). However, standard curves prepared from *l*- and *dl*-aspartic acid, respectively, are not superimposable in the upper regions. It is necessary, therefore, to use *l*-aspartic acid as the standard in assays. In the case of serine, however, only the *l* enantiomorph is available to *Lactobacillus delbrückii*, since the *dl* form is only half as active as the *l* isomer.[2] Standard curves prepared with *l*- and *dl*-serine are identical. Therefore, the *dl* form, which is the more readily available one, can be used as the standard.

TABLE II

Aspartic Acid and Serine Content of Gelatin at Different Assay Levels

Amount of gelatin per assay tube	Aspartic acid		Amount of gelatin per assay tube	Serine	
	Found	Content		Found	Content
mg	*mg*	*mg per gm.*	*mg*	*mg*	*mg. per gm.*
2	0.105	52.5	3	0.122	40.7*
3	0.160	53.3	4	0.146	36.5
4	0.205	51.3	5	0.172	34.4
6	0.330	55.0	6	0.210	.33.5
8	0.385	48.1	7	0.250	35.7
Average	.	52.0			35.0

* Omitted from the average.

DISCUSSION

The *Lactobacillus delbrückii* method for determining aspartic acid and serine in purified proteins satisfies the usual criteria of reliability in that (*a*) assay values obtained from different dosage levels of test samples agree closely, indicating absence of stimulatory or inhibitory substances (Table II); (*b*) there is good duplication of assay values on the same protein in different experiments involving different operators and preparation of fresh hydrolysates (Table III); (*c*) recoveries of aspartic acid and serine added to proteins prior to hydrolysis are quantitative within the experimental error of microbiological methods, namely, ±15 per cent (Tables IV and V).

In view of the wide discrepancies in the literature on the percentages of individual amino acids in various proteins, arrived at by chemical methods, it is not profitable to compare such values too closely with those obtained by microbiological assay. It is evident, however, that the microbiological values for aspartic acid and serine of practically all of the proteins investigated are in good agreement with those arrived at by recent, improved chemical methods (Table VI). This is true for the percentages of aspartic acid in β-lactoglobulin and egg albumin, which are in close accord with those obtained by Chibnall by highly refined chemical techniques. The microbiological values of 14.5 per cent for the serine content of silk fibroin is reasonable when compared to 9 per cent by isolation, admittedly incomplete (19), and agrees fairly well with the 13.6 per cent of Nicolet and Shinn obtained with their periodate method. The latter applies also to gelatin, which contains 3.6 or 3.8 per cent serine by microbiological assay compared to 3.3 per cent found by the periodate method.

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[2] Kindly supplied by the late Dr. Max Bergmann.

TABLE III

Reproducibility of Assays

The results are in per cent, on a dry basis.

Protein	Aspartic acid				Serine			
	Assay 1	Assay 2	Assay 3	Mean	Assay 1	Assay 2	Assay 3	Mean
Casein, S. M. A	5.7	6.2	6.4	6.1	6.4	6.3		6.4
Gelatin, Knox	5.4	5.8	5.9	5.7	3.7	3.9		3.8
β-Lactoglobulin	9.3	8.8	9.7	9.3	3.1	3.3	3.1	3.2

TABLE IV

Recovery of Aspartic Acid

Material	Aspartic acid content	Aspartic acid added	Total	Aspartic acid recovered	Recovery
	mg per gm	*mg per gm*	*mg. per gm.*	*mg per gm*	*per cent*
Casein	57.7	16.7	74.4	73.6	99
	57.7	66.6	124.3	129.7	104
Gelatin (Bacto)	72.5	16.7	89.2	103.5	116
	72.5	66.6	139.1	150.0	108

TABLE V

Recovery of Serine

Material	Serine content	Serine added	Total	Serine recovered	Recovery
	mg per gm	*mg per gm.*	*mg per gm.*	*mg. per gm.*	*per cent*
Casein ..	59.0	16.7	75.7	77.3	102
	59.0	41.8	100.8	104.3	103
Gelatin (Bacto)	24.7	16.7	41.4	44.7	108
	24.7	41.8	66.5	69.0	104

In a few tests on the specificity of the response of *Lactobacillus delbrückii* to aspartic acid, malic and fumaric acids were found to have about 1 per cent the activity of aspartic acid when assayed with small quantities of the latter but were completely inactive when used alone. Succinic acid was inactive under both sets of conditions. Asparagine is as active as aspartic acid. The fact that *Lactobacillus delbrückii* differentiates completely between serine and aspartic acid and the structurally closely related amino acids in the basal medium (see Table I) indicates a considerable degree of specificity of the microbiological method. The latter is supported also by the good agreement between the microbiological and recent chemical data.

As stated previously, most of the experimental work was completed before pyridoxamine became available and its stimulatory effect on growth with serine determined. However, the serine values of the proteins listed in Table VI did not change on reassay in media containing pyridoxamine, presumably because the proteins were free of pyridoxamine.

TABLE VI

Aspartic Acid and Serine Content of Proteins

The results are in per cent, calculated for oven-dried (105°) material

Protein	Aspartic acid		Serine	
	By microbiological assay	Chemical value in literature	By microbiological assay	Chemical value in literature
Casein, S. M. A.	6.1	6.0 (14)	6.4	5.0 (17)
Gelatin, Silver Label	5.5	3.4 (15)	3.6	3.3 (17)
" Knox	5.7		3.8	
Silk fibroin*			14.5	13.6 (18)
β-Lactoglobulin*	9.3	9.9 (16)	3.2	4.3 (17)
Egg albumin*	8.0	8.1 (16)		

* We are indebted to the late Dr. Max Bergmann for these proteins.

Preliminary experiments indicate that the *Lactobacillus delbrückii* method cannot be applied to the assay of aspartic acid or serine in natural products such as wheat or rye without modification because of toxicities encountered at the higher dosage levels with hydrolysates of such substances.

SUMMARY

An accurate and specific microbiological method is described for the determination of aspartic acid and serine in purified proteins. It is based on the quantitative response of *Lactobacillus delbrückii* to those two amino acids as measured by titration of the lactic acid formed.

BIBLIOGRAPHY

1. Hutchings, B. L., and Peterson, W. H., *Proc. Soc. Exp. Biol. and Med.*, **52**, 36 (1943).
2. Shankman, S., *J. Biol. Chem.*, **150**, 305 (1943).
3. Kuiken, K. A., Norman, W. H., Lyman, C. M., Hale, F., and Blotter, L., *J. Biol. Chem.*, **151**, 615 (1943)
4. Hegsted, D. M., *J. Biol. Chem.*, **152**, 193 (1944).
5. Schweigert, B. S., McIntire, J. M., Elvehjem, C. A., and Strong F. M., *J. Biol. Chem.*, **155**, 183 (1944).
6. McMahan, J. R., and Snell, E. E., *J. Biol. Chem.*, **152**, 83 (1944).
7. Greene, R. D., and Black, A., *J. Biol. Chem.*, **155**, 1 (1944).
8. Dunn, M. S., Camein, M. N., Rockland, L. B., Shankman, S., and Goldberg, S. C., *J. Biol. Chem.*, **155**, 591 (1944)
9. Shankman, S., Dunn, M. S., and Rubin, L. B., *J. Biol. Chem.*, **151**, 511 (1943).
10. Hutchings, B. L., and Peterson, W. H., *Proc. Soc. Exp. Biol. and Med.*, **52**, 36 (1943).
11. Snell, E. E., *J. Biol. Chem.*, **154**, 313 (1944).
12. Harris, S. A., Heyl, D., and Folkers, K., *J. Biol. Chem.*, **154**, 315 (1944).
13. Stokes, J. L., and Gunness, M., *J. Biol. Chem.*, **154**, 715 (1944).
14. Cohn, E. J., and Edsall, J. T., Proteins, amino acids and peptides, American Chemical Society monograph series, New York, 354 (1943).
15. Dakin, H. D., *J. Biol. Chem.*, **44**, 499 (1920).
16. Chibnall, A. C., *Proc. Roy. Soc. London, Series B*, **131**, 136 (1942).
17. Nicolet, B. H., and Shinn, L. A., *J. Biol. Chem.*, **139**, 687 (1941).
18. Nicolet, B. H., and Saidel, L. J., *J. Biol. Chem.*, **139**, 477 (1941).
19. Stein, W. H., Moore, S., and Bergmann, M., *J. Biol. Chem.*, **139**, 481 (1941).

THE BIOLOGICAL CONVERSION OF CHOLESTEROL TO PREGNANEDIOL*

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The animal organism is capable of synthesizing the steroids which it normally requires. It is conceivable that the compounds possessing the cyclopentanophenanthrene structure arise either individually by synthesis or by degradation from a common precursor. If a parent substance exists from which the various steroids are derived, it might well be cholesterol, which is widely distributed in animal tissues and can be converted *in vitro* into derivatives of bile acids and steroid hormones.

Deuterio cholesterol, which has been prepared in this laboratory (1), provides a suitable tool for investigating *in vivo* relationships of steroids. With the aid of cholesterol containing deuterium both in the isooctyl side chain and in the nuclear moiety of the molecule, evidence has been secured of the direct transformation of cholesterol to cholic acid in the dog (2).

An experiment has now been carried out in order to ascertain whether a steroid hormone may be similarly related to cholesterol. As deuterium analysis by the present methods requires much larger quantities of hormones than are obtainable from tissues or urine of the small laboratory animals, advantage has been taken of the relatively large excretion of pregnanediol in the later stages of human pregnancy. Pregnanediol, while not a steroid hormone in the sense that it is biologically active, is recognized to be a product of progesterone metabolism. The conclusions drawn from the results of this experiment may therefore be inferentially applied to progesterone.

Deuterio cholesterol was taken by a woman in the 8th month of pregnancy. At this stage pregnanediol glucuronidate can be isolated in amounts sufficient for deuterium analysis from a single day's urine. From the urine excreted during the experimental period and for 3 days thereafter, pregnanediol was isolated as the sodium glucuronidate and analyzed for deuterium. Significant isotope concentrations were present in these samples, showing clearly that pregnanediol had been formed directly from

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cholesterol. By comparison of these data with the deuterium concentration of cholesterol in the blood, it became evident that the major part of the pregnanediol formed during the experimental period had arisen by degradation of cholesterol.

EXPERIMENTAL

The deuterio cholesterol was prepared by exchange with D_2O and deuterio acetic acid in the presence of active platinum (1). It contained 4.18 atom per cent excess deuterium. M.p. 148–149° (corrected); $\alpha_D = -37.5°$ (1.5 per cent in $CHCl_3$).

9 gm. of deuterio cholesterol were dissolved in hydrogenated vegetable oil (Nutola) and administered to a woman in the 8th month of pregnancy. The cholesterol was taken in 1 gm. doses three times daily over a period of 3 days. During this time and during the 3 following days the subject ate a diet otherwise cholesterol-free in order to prevent dilution of deuterio cholesterol by the non-isotopic cholesterol present in normal food. Urine collection was started immediately after the first intake of deuterio cholesterol and was continued for 6 days. Pregnanediol-3(α),20(α) was isolated separately from each 24 hour urine collection as sodium pregnanediol glucuronidate according to Venning (3). The amounts of glucuronidate obtained from 24 hour urine samples were, after two reprecipitations from water-acetone, 83, 94, 100, 60, 102, and 61 mg. One to two recrystallizations from 90 per cent ethanol yielded colorless preparations which had a melting point of 273–277° (uncorrected). On drying *in vacuo* at 137° the samples lost 6.2 to 7.0 per cent of their weight; calculated for $2H_2O$, 6.5 per cent. The anhydrous sodium pregnanediol glucuronidate is hygroscopic and takes up 2 moles of water when exposed to air. The identity of one of the samples (No. 5 in Table I) was checked by elementary analysis.

$C_{27}H_{43}O_8Na$.	Calculated.	C 62.55,	H 8.36,	Na 4.44
	Found.	" 62.94,	" 8.55,	" 4.40
		" 62.85,	" 8.60,	" 4.24

The deuterium analyses were carried out in most cases with the sodium pregnanediol glucuronidate samples. The values, calculated for free pregnanediol, are given in Table I.

Hydrolysis of Sodium Pregnanediol Glucuronidate—52 mg. of sodium pregnanediol glucuronidate (Sample 4) were hydrolyzed by heating under a reflux in a mixture containing four parts of ethanol and one part of 10 per cent hydrochloric acid. The reaction mixture was distributed between water and ether; the ether layer was washed free of acid and brought to dryness. The residue after recrystallization from acetone-ethanol 3:1 gave

12 mg. of crystalline material, m.p. 235–237° (corrected), unchanged on admixture of an authentic sample of pregnanediol.[1] It contained 0.11 atom per cent excess deuterium in accord with the values calculated from the deuterium analysis of the glucuronidates. This agreement indicates that in the isolated sodium pregnanediol glucuronidate only the steroid moiety contained deuterium.

Although contamination of the excreted glucuronidate by deuterio cholesterol seemed unlikely, it was thought desirable to eliminate this possibility. The combined mother liquors from the recrystallizations of the sodium pregnanediol glucuronidate Samples 3 to 6 were hydrolyzed by ethanolic HCl and the unsaponifiable fraction precipitated by digitonin in 90 per cent EtOH solution. The material which was not precipitated by digitonin was dissolved in benzene and passed through a column of activated aluminum oxide. The fractions eluted by benzene containing 10 to 30 per cent acetone were combined, passed again through a column of aluminum oxide, and eluted by benzene-acetone 5:1. This fraction, after successive recrystallizations from acetone, 70 per cent ethanol, and methanol, yielded 18 mg. of crystalline pregnanediol, m.p. 231–233° (corrected). It contained 0.106 atom per cent excess deuterium (micromethod).

TABLE I

Deuterium Concentrations in Pregnanediol-3(α),20(α) after Ingestion of Deuterio Cholesterol Containing 4.18 Atom Per Cent Excess D_2

Day of experiment*	Atom per cent excess deuterium	
	In pregnanediol†	In blood cholesterol
1, 2	0.010	0.234
3	0.100	
4	0.110‡§	
5	0.110§	
6	0.097	

* Deuterio cholesterol was administered during the first 3 days. Urine collection was continued during the 3 following days.

† Calculated from the deuterium content of pregnanediol sodium glucuronidate.

‡ Analyzed as free pregnanediol.

§ Micromethod.

$C_{21}H_{36}O_2$ Calculated. C 78.68, H 11.33
Found. " 78.89, " 11.26

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[1] We are indebted to Dr. H. Hirschmann, Department of Medicine, Western Reserve University, Cleveland, Ohio, and to Dr. M. M. Hoffman, University Clinic, Royal Victoria Hospital, Montreal, Canada, for supplying samples of pregnanediol.

Blood Cholesterol—12 hours after the last ingestion of deuterio cholesterol (4th day of experiment) a sample of blood was taken (12 ml.). After hydrolysis by alcoholic KOH cholesterol was precipitated as the digitonide from the unsaponifiable fraction. The cholesterol digitonide (120 mg.) contained 0.078 atom per cent D_2, whence the cholesterol must have contained 0.234 per cent.

The author is greatly indebted to Dr. D. Rittenberg for micro deuterium analyses carried out by an unpublished method.

DISCUSSION

The isotope concentration in the excreted pregnanediol reached a maximum value of 0.11 per cent on the 4th day. The cholesterol from a sample of blood secured 12 hours after the last cholesterol ingestion contained 0.23 per cent D_2. In order to determine the extent of the cholesterol-pregnanediol conversion it is necessary to consider the maximum deuterium concentration attainable in pregnanediol on the assumption that it was derived from cholesterol as the only source. This maximum value will depend largely on two factors; *viz.*, the deuterium concentration of the sterol which served as the immediate precursor of progesterone and on the loss of deuterium caused by the chemical reactions which are involved in the conversion of cholesterol to progesterone and pregnanediol respectively. The value of 0.23 per cent D_2 (calculated for carbon-bound hydrogen only, 0.24 per cent) in blood cholesterol results from dilution in the organism of the dietary deuterio cholesterol (4.18 per cent D_2) by the normal cholesterol of tissues and body fluids. As the labeled cholesterol presumably reached the site of hormone formation primarily by way of the blood stream, it may be assumed that this value represents the isotope concentration in the cholesterol available for intermediary reactions.

The degradation of cholesterol to pregnanediol will, irrespective of the reaction mechanism, lead to loss of carbon-bound deuterium from the nuclear portion of the sterol molecule. During the breakdown of cholesterol to progesterone 2 atoms of isotopic hydrogen are lost as a result of oxidation at carbon atoms 3 and 20. Conversion of this unsaturated diketone to pregnanediol involves the uptake from the body fluids of 6 non-isotopic hydrogen atoms, 4 in the reduction of the two ketonic groups and 2 in the saturation of the double bond. In addition to the hydrogen of the 3-hydroxyl group, which was of normal composition in the original deuterio cholesterol, a total of 5 non-isotopic hydrogen atoms must have been introduced into the steroid molecule during the degradation reactions. Of the 36 hydrogen atoms in pregnanediol not more than 30 can be isotopic. Hence, the deuterium concentration in the isolated pregnanediol could have reached at most 83 per cent of that contained in the sterol from which it was formed. For the reasons cited above, the hormone precursor could have contained at most 0.24 per cent of its carbon-bound hydrogen as deuterium, the pregnanediol therefore not more than 0.20 per cent. The value actually found in the pregnanediol, which was excreted immediately before and after the blood sample had been taken, was 0.11 per cent, *i.e.* 55 per cent of the theoretical maximum.

These calculations are based on the assumption that in deuterio cholesterol deuterium is equally distributed over side chain and nucleus of the molecule. In deuterio cholesterol prepared previously under identical conditions, the average isotope concentration of the nuclear hydrogen atoms was found to be 25 per cent less than that of the hydrogen in the isooctyl side chain (1). If this were the case also in the cholesterol used in the present experiment, then the above value of 55 per cent for the sterol-pregnanediol conversion would be raised to 68 per cent.

The present result, in conjunction with our earlier finding on the *in vivo* transformation of cholesterol to cholic acid, provides experimental support for the hypothesis that bile acids and steroid hormones can be formed from cholesterol as a common precursor. This implies that the animal organism can carry out reactions which lead to shortening of the cholesterol side chain, the introduction of hydroxyl groups into the side chain and into the nucleus, and to epimerization of preexisting hydroxyl groups. Analogous experimental demonstrations of relationships between cholesterol and hormones other than progesterone do not yet appear feasible.

The present findings make it possible to attribute to cholesterol specific functions in intermediary metabolism, though the quantities of cholesterol apparently utilized for conversion to bile acids and steroid hormones are negligible as compared to those present in animal tissues In some tissues such as brain and nerve which are very rich in cholesterol, this sterol appears to be metabolically inert; *i.e.*, it is not regenerated at a detectable rate (4, 2). Its function in these tissues can therefore not be associated with reactions requiring continuous breakdown and replacement.

The author wishes to thank Mr. William Saschek of this Department and Mr. J. Alicino, of The Squibb Institute for Medical Research, for elementary analyses.

SUMMARY

1. Deuterio cholesterol containing 4.18 atom per cent excess deuterium was administered to a woman in the 8th month of pregnancy. From the urine pregnanediol-3(α), 20(α) was isolated as sodium pregnanediol glucuronidate and found to contain significant concentrations of deuterium.

2. The isotope concentration in pregnanediol was of the same order of magnitude as that of cholesterol circulating in the blood. From these data it can be estimated that one-half to two-thirds of the pregnanediol excreted arose by degradation of cholesterol.

3. These findings suggest that direct conversion of cholesterol to progesterone is a normal process.

BIBLIOGRAPHY

1. Bloch, K., and Rittenberg, D., *J. Biol. Chem.*, **149**, 505 (1943).
2. Bloch, K., Berg, B. N., and Rittenberg, D , *J. Biol. Chem.*, **149**, 511 (1943).
3. Venning, E. H., *J. Biol. Chem.*, **126**, 595 (1938).
4. Waelsch, H., Sperry, W. M., and Stoyanoff, V. A., *J. Biol. Chem.*, **135**, 291 (1940).

LACTIC ACID OXIDATION QUOTIENT IN MINCED BRAIN OF NORMAL AND AVITAMINOTIC CHICKEN

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Few data are available on the quotient of lactic acid oxidation, (lactic acid removed)/(lactic acid oxidized), in the central nervous system. Warburg, Posener, and Negelein (1), working with gray substance of rat cerebral slices, found a value of 4.8. Holmes and Ashford (2, 3) obtained quotients ranging generally between 1.4 and 5.6 in minced rabbit brain.

The determination of the quotient of lactic acid oxidation in birds seemed particularly interesting because these animals show a disturbance in the oxidation of lactic acid in specific regions of the central nervous system (4–6). The first determinations in bird brain were performed by Galvão and Pereira (7) who measured the uptake of O_2, both with and without the addition of lithium lactate, as well as lactate removal. In these investigations ground cerebrum and brain stem from normal and B_1 avitaminotic chickens were studied. With the normal birds the quotients were of the same order as those determined by Meyerhof (8) for the skeletal muscle. With birds showing pronounced symptoms of B_1 avitaminosis, the decreased oxidation of the lactic acid formerly noted for the brain stem was not accompanied by an impaired removal of lactate. The quotient of lactic acid oxidation, therefore, increased. Addition of vitamin B_1 produced a partial restoration of the lactate oxidation, without significant influence on the removal of lactate; the quotient, consequently, tended toward normal.

In the present experiments the nervous tissue employed was finely minced. This treatment of the nervous tissue brought about results substantially different from those obtained with thoroughly ground tissue used in the previous experiments.

Methods

Adult normal and B_1 avitaminotic chickens were used, the avitaminotic birds being fed polished rice. Immediately after decapitation the cerebrum and brain stem were isolated and finely minced with scissors, the temperature of the materials used being kept at 38°. The uptake of O_2 in 2 hours was measured by Warburg's manometric technique as described in a previous publication (9). Two homogeneous suspensions of the same tissue, each of 60 mg. of wet weight in 1 cc. of Ringer's solution, according to Warburg's directions, were rapidly prepared. One of these suspensions was used as prepared; to the other was added a known quantity of lithium lactate (2 to 5 mg.). Sometimes a third suspension was made containing, in addition to lactate, vitamin B_1 (betaxin, Bayer). From the extra O_2 the amount of lactic acid oxidized was calculated, with the factor 1.34.

The removal of the lactic acid was determined by establishing the difference between the amount added plus the preformed lactic acid and that found in the vessels after the determination of the O_2 uptake. The tissue suspensions were deproteinized by the Schenck method according to Lehnartz's (10) modification, and lactic acid was determined by the iodometric permanganate method. It was possible to increase both yield and precision by means of the modifications we introduced (11). Under such conditions the errors in the determination of lithium lactate did not exceed 0.03 mg.

EXPERIMENTAL AND DISCUSSION

Tables I to IV show the values of O_2 uptake without addition of lactate (O_2 basal uptake), with addition of lithium lactate (O_2 uptake in lactate), the difference between these uptakes (extra O_2 uptake), the lactic acid removal, and the oxidation quotient. All the data are calculated on a basis of 2 hours and 100 mg. of wet weight.

Comparing the present results obtained with the finely minced nervous tissues with those previously published (7) in which thoroughly ground tissue was used, one notices a great increase of all these values, with the exception of the oxidation quotient, which remains practically of the same magnitude.

Cerebrum—Tables I and II show that there is no decrease of the basal uptake of O_2 and of the extra O_2, nor is there any significant alteration of the lactate uptake and oxidation quotient in the cerebrum of birds with beriberi as compared with normal birds, facts already noted in the case of ground cerebrum.

Brain Stem—Tables III and IV summarize the results obtained from normal chickens and from others with definite symptoms of beriberi. In addition the effects of adding vitamin B_1 in the presence of lactate are noted. Inspection of these data leads to the conclusion that in avitaminotic birds an alteration of basal oxygen uptake did not occur, but instead a great diminution of extra O_2 was found. Not only was the lactate not oxidized but in one instance (Chicken 5) it even produced an inhibition of the residual respiration. The addition of vitamin B_1 effected a certain degree of restoration of the O_2 uptake in the presence of lactate. All these observations are in agreement with those we had previously reported on ground brain stem.

The result which strikes us as the most interesting, however, and to which we desire to draw particular attention, is the profound diminution of the lactate removal in the brain stem of the chickens suffering from B_1 avitaminosis. Chicken 5 showed a great decrease of the lactic acid removal. The decrease of the extra O_2 uptake was, however, even greater than the impairment in the removal of lactic acid; hence, the oxidation quotient was high. Although a diminution of the extra O_2 uptake in the presence of lactate occurred in the previous experiments of ground brain stem, yet no such decrease of lactic acid utilization was found, nor was there an improvement in the lactic acid removal on the addition of vitamin B_1, an improvement which tended to reduce the high oxidation quotient. These results disclose essential differences due to the two methods of preparation.

TABLE I

Minced Cerebrum (Normal Chicken); Respiration and Oxidation Quotient of Lactic Acid (in 2 Hours and per 100 Mg., Wet Weight)

Chicken No	O_2 basal uptake	O_2 uptake in lactate	O_2 extra uptake	Lactic acid removed	Oxidation quotient
	c mm.	c.mm.	c mm.	mg.	
8	220.1	410.0	189.9	0.668	2.6
9	257.7	383.3	125.6	0.582	3.5
10	248.6	435.5	186.9	0.643	2.6
11	245.0	374.8	129.8	0.551	3.4
12	234.3	364.0	129.7	0.630	3.7
13	274.4	455.4	181.0	0.787	3.3
Average	246.6	403.8	157.2	0.643	3.1

TABLE II

Minced Cerebrum (B_1 Avitaminotic Chicken); Respiration and Oxidation Quotient of Lactic Acid (in 2 Hours and per 100 Mg., Wet Weight)

Chicken No	O_2 basal uptake	O_2 uptake in lactate	O_2 extra uptake	Lactic acid removed	Oxidation quotient
	c mm	c mm	c mm.	mg.	
1	290.3	424.0	133.7	0.496	2.8
4	272.6	511.8	239.2	0.557	1.7
5	286.4	393.5	107.1	0.381	2.7
8	340.4	440.1	99.7	0.810	6.0
Average	297.4	442.3	144.9	0.561	3.3

It might be interesting to compare our observations with those of Meiklejohn (12), which greatly influenced the later investigations on the mechanism of action of vitamin B_1. Using only minced cerebrum of pigeons, Meiklejohn suggested that the lactate removal in avitaminosis B_1 and in normal birds is of the same order. This result coincides with that obtained in our previous experiments (7) in which thoroughly ground cerebrum or brain stem was employed. In our present experiments with finely minced nervous tissue, however, we noted a great reduction of the lactate removal by brain stem but not by cerebrum.

TABLE III

Minced Brain Stem (Normal Chicken); Respiration and Oxidation Quotient of Lactic Acid (in 2 Hours and per 100 Mg., Wet Weight)

Chicken No.	O_2 basal uptake	O_2 uptake in lactate	O_2 extra uptake	Lactic acid removed	Oxidation quotient
	c.mm.	*c mm*	*c. mm.*	*mg.*	
1	184.8	267.6	82.8	0.512	4.6
2	213.6	336.0	122.4	0.346	2.1
4	202.8	366.0	163.2	0.679	3.1
5	231.6	369.6	138.0	0.487	2.6
6	219.6	415.2	195.6	0.604	2.3
8	211.6	386.2	174.6	0.683	2.9
9	244.9	371.9	127.0	0.531	3.1
11	195.0	322.6	127.6	0.551	3.2
Average. .	213.0	354.4	141.4	0.549	3.0

TABLE IV

Minced Brain Stem (B_1 Avitaminotic Chicken); Respiration and Oxidation Quotient of Lactic Acid, with and without Addition of Vitamin B_1 (in 2 Hours and per 100 Mg., Wet Weight)

Chicken No	Symptoms*	O_2 basal uptake	O_2 uptake in lactate	O_2 uptake in lactate + vitamin B_1	O_2 extra uptake	O_2 extra + vitamin B_1	Lactic acid removed	Lactic acid removed with vitamin B_1 added	Quotient without vitamin B_1	Quotient with vitamin B_1
		c. mm.	*c mm.*	*c. mm*	*c mm.*	*c. mm*	*mg*	*mg.*		
1	+	254.6	280.5		25.9					
2	+	197.0	262.3	276.3	65.3	79.3	0.381	0.385	4.4	3.6
3	+	212.7	265.7	275.0	53.0	62.3	0.367		5.2	
4	++	187.0	259.3	299.6	72.3	112.6	0.285	0.586	2.9	3.9
5	++	197.1	179.3	196.9	−17.8	−0.2	0.388	0.186	∞	∞
6	+++	220.3	224.5		4.2		0.340		∞	
7	++	257.7	286.3		28.6		0.213		5.6	
8	++	222.5	287.5	341.5	65.0	119.0	0.443	0.605	5.5	3.6
Average		218.6	255.6	277.9	37.0	74.6	0.345	0.441		

* +, contracture of legs; ++, contracture of legs and opisthotonus, either slight or appearing only on excitation; +++, contracture of legs and continuous and strong opisthotonus.

Meiklejohn's experiments were conducted on cerebrum; yet it is known that in birds the chemical lesion of B_1 avitaminosis is located predominantly in the brain stem. This location of the lesion was again observed in the present experiments, for no difference in the extra O_2 was found between normal and avitaminotic cerebral tissues.

Meiklejohn, using the cerebral tissues of pigeons with B_1 avitaminosis, found that the addition of vitamin B_1 produced an increase of the O_2 uptake. Our own experiments disclosed an increase of extra O_2 in the brain stem both with ground and with minced tissue in the presence of lactate and vitamin B_1. Moreover, in contrast with our former experiments the present ones suggest an increased removal of lactate.

No close relationship exists between the extra O_2 uptake in the presence of lactate and the lactate removal, since the oxidation quotients are different in the normal and the avitaminotic brain stem. The lack of a parallelism between O_2 uptake and lactate removal is also evident in other experiments now in progress.

SUMMARY

Determinations were made of O_2 uptake with and without lactate, lactate removal, and oxidation quotient of lactic acid, in finely minced cerebrum and brain stem of normal and B_1 avitaminotic chickens.

In the avitaminotic brain stem, a great diminution of extra O_2 was accompanied by a considerable though smaller reduction in lactate removal; consequently, the oxidation quotient tended to rise. Vitamin B_1, besides promoting the extra O_2 uptake, also increased lactate removal.

No differences were found between normal and B_1 avitaminotic cerebrum.

The authors emphasize the difference of behavior between thoroughly ground and finely minced nervous tissue.

BIBLIOGRAPHY

1. Warburg, O., Posener, K., and Negelein, E., *Biochem. Z.*, **152**, 309 (1924).
2. Holmes, E. G., and Ashford, C. A., *Biochem. J.*, **24**, 1119 (1930).
3. Ashford, C. A., and Holmes, E G., *Biochem. J.*, **25**, 2028 (1931).
4. Galvão, P. E., and Florence, C. H , *An. Paulistas med. e cir.*, **3**, 201 (1932).
5. Galvão, P. E., and Florence, C. H., *Arch. ges. Physiol.*, **233**, 714 (1934).
6. Gavrilescu, N., Meiklejohn, A. P., Passmore, R., and Peters, R. A., *Proc. Roy. Soc., London, Series B*, **110**, 431 (1932).
7. Galvão, P. E., and Pereira, J., *Arq. inst. biol.*, **11**, 139 (1940).
8. Meyerhof, O., Die chemischen Vorgänge im Muskel, Berlin (1930).
9. Galvão, P. E., and Pereira, J., *Biochem. Z.*, **289**, 136 (1936).
10. Lehnartz, E., *Z. physiol. Chem.*, **179**, 1 (1928).
11. Galvão, P. E., Florence, C. H., and Pereira, J., *Arq. inst. biol.*, **9**, 39 (1938).
12. Meiklejohn, A. P., *Biochem. J.*, **27**, 1310 (1933).

PYRUVIC ACID

III. THE LEVEL OF PYRUVIC AND LACTIC ACIDS, AND THE LACTIC-PYRUVIC RATIO, IN THE BLOOD OF HUMAN SUBJECTS. THE EFFECT OF FOOD, LIGHT MUSCULAR ACTIVITY, AND ANOXIA AT HIGH ALTITUDE*

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It is now generally agreed that the metabolism of carbohydrate results in the production of pyruvic acid, whose further metabolism requires oxygen and diphosphothiamine. Lactic acid is derived by reduction, and large quantities are produced under anaerobic conditions. These metabolites accumulate (*a*) during increased breakdown of carbohydrate, (*b*) in anoxia, and (*c*) in thiamine deficiency. The determinations of pyruvic and lactic acids are useful in studies dealing with many phases of carbohydrate metabolism.

The rise of these acids under a given condition is often small and may be overlooked unless the results are compared with those which have been obtained in some standard state. It is customary, therefore, to take blood only from the fasting resting subject. Unfortunately, this limits the usefulness of the methods and prevents their application to many clinical and physiological problems. The performance of fasting subjects is often poor in certain tests, especially when they are conducted at high altitude (unpublished results). The basal state not only is difficult to attain, but may be undesirable.

In previous papers, simple procedures were described for sampling of blood and the determination of pyruvic and lactic acids (1–3). In the present paper, we shall present data showing the effect of (*a*) food, (*b*) muscular activity, and (*c*) anoxia, the three most important factors which affect the concentration of pyruvic and lactic acids in the normal healthy subject. It will be shown that light muscular activity, even a brisk walk, has little effect (4–7), and the levels of the acids rise and return to the basal levels within 4 hours or less after a meal.[1] It is evident that the

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[1] This result was anticipated on the basis of the work of Trimble and Maddock (8), Haggard and Greenberg (9), and others who had shown that the sugar of the blood is restored to the original or basal level 3 to 4 hours after a meal.

energy for the usual daily activities can be obtained entirely by oxidation without accumulation of acids. On the basis of these data we have devised a procedure which yields minimum and constant results, and which can be used in the clinic and the laboratory under conditions more nearly approaching the physiological optimum than the fasting state. Our study of the third factor, anoxia at high altitude, not only provides a means of applying the technique to a problem of great present interest, but also indicates the degree of anoxia at which lactic and pyruvic acids begin to accumulate in the blood.

EXPERIMENTAL

The general procedure was as follows: The patient, or experimental subject, ate the usual breakfast or noon meal. He was cautioned against performing any strenuous work or exercise, running, walking up steps, and further ingestion of food of any kind. He came to the laboratory 3 hours after the meal, and sat upright in a low, cushioned easy chair ½ hour or longer before the sample of blood was taken.

The procedure was slightly altered if lactic acid was to be determined. As will be shown, the pyruvic acid level is not affected by long rest without activity; however, the level of lactic acid may rise. If the subject had rested more than 30 minutes, he was asked to walk slowly about the laboratory for a minute or so, and then to sit down again. Blood was taken 5 to 10 minutes later.

The tourniquet was lightly applied, and was released as soon as the needle had entered the vein; the relaxed forearm was rested on the table or on the arm of the chair. *The subject was cautioned against clenching the hands before or during the collection of blood.*

If an experiment required several hours for completion, the meal was eaten at 6 or 11 a.m.

Blood was collected and measured as previously described (1). It was analyzed for pyruvic acid by the modified Lu (10) procedure of Friedemann and Haugen (2). Ethyl acetate solvent was used throughout. Lactic acid was determined by the method of Shaffer (3). For the determination of blood sugar, a separate sample was precipitated by the Somogyi $Zn(OH)_2$ method (11) and analyzed by the method of Shaffer, Hartmann, and Somogyi (12).

Effect of Food

Bueding and coworkers (13, 14) have adequately described the effect of carbohydrate on the pyruvic acid content of the venous blood of resting subjects. To our knowledge, similar observations, together with simultaneous determinations of lactic acid, have not been made in individuals while they are performing their daily tasks.

Mixed Diet—A group of ten subjects, consisting of laboratory and office workers, received breakfast at 8 a.m. and lunch at 12 noon. Blood was taken at hourly intervals throughout the day, during which the subjects went about their usual work. The composition of the meals, the hours of rest and work, and other details are given in Table I. The results from four subjects which are shown in Table I were typical of the types of response which may be expected from a mixed diet.

TABLE I

Variation of Pyruvic and Lactic Acid Content of Blood During Working Day

The subjects, women laboratory workers, received breakfast and lunch in the laboratory. The breakfast consisted of cereal with cream, two slices of toasted bread, butter, two eggs, milk, and fruit. The lunch consisted of meat, vegetable, two slices of bread, salad with salad dressing, milk and butter. The energy content of the noon meal was approximately 850 calories.

Time of blood collection	Experimental conditions	M K			E G			G E. H.		J. R	
		Pyruvic acid	Lactic acid	Lactic/Pyruvic	Pyruvic acid	Lactic acid	Lactic/Pyruvic	Pyruvic acid	Lactic acid	Pyruvic acid	Lactic acid
a m		*mg per cent*	*mg per cent*		*mg per cent*	*mg per cent*		*mg per cent*	*mg. per cent*	*mg. per cent*	*mg per cent*
8	After 15–20 min. rest	0.94	12.2	13.0	0.73	11.5	15.8	0.76	12.0	0.59	9.0
8.30	Breakfast, 8.05–8.20, rest continued to 8.30	1.04	14.3	13.7	1.16	17.0	14.7	0.73	9.8	0.68	12.0
9	Laboratory work begun at 8.30	1.06	12.7	12.0	1.07	17.1	16.0	0.83	10.7	0.74	9.9
10	" "	1.26	14.3	11.4	0.80	11.5	14.4	1.07	15.9	0.67	10.5
11	" "	1.12	13.6	12.1	0.61	11.3	18.5	0.77	11.7	0.66	10.1
12	Lunch 12–12.30, rest continued to 1 p.m.	0.77	10.1	13.1	0.57	8.9	15.6	0.76	10.1	0.76	11.4
p m											
1	Laboratory work begun at 1 p.m.	1.29	15.8	12.2	1.29	19.0	14.7	0.94	14.6	0.83	11.1
2	" "	1.35	16.3	12.1	1.03	15.3	14.9	0.94	10.9	0.61	8.6
3	" "	1.24	12.0	9.7	1.02	15.0	14.7	0.76	10.5	0.70	10.5
4	" "	0.91	10.9	12.0	0.56	9.1	16.3	0.77	10.1	0.56	7.9
5	" "	0.91	9.6	10.6	0.44	8.1	18.3	0.57	8.9	0.76	9.0

Considerable variation in results is shown between individuals: (*a*) a prompt, marked rise of pyruvic acid may be obtained after each meal (M. K., E. G.); (*b*) it may be delayed (G. E. H.); or (*c*) it may not be observed at all (J. R.). The last result is probably exceptional, since it was observed in only one of the ten subjects; it was not observed in this subject in a subsequent experiment. Such differences were noted also in the results from E. G., Tables I and II.

Following the ingestion of a meal, the pyruvic and lactic acid levels of the blood rose and reached a maximum in 1 to 2 hours, after which they rapidly declined. Low and relatively constant levels, which varied within the limits of the fasting resting levels, were attained between the 3rd and 4th hours.

The rate of rise and decline of pyruvic acid roughly paralleled the fluctuations of the sugar concentration (Tables II and III). However, the height of the sugar level was not necessarily related to that of pyruvic acid (subject A, Table II; E. S., Table III).

Protein and Fat—Since protein is largely converted into dextrose and since it has a marked specific dynamic action on metabolism it is possible that the rise of pyruvic acid, following a mixed meal, may be due not only to the increase of sugar metabolism, but also to the increased production of keto acids from amino acids. Two subjects ate 455 gm. of roast turkey meat. This quantity of meat contained approximately 110 gm. of protein and 30 gm. of fat. Assuming a maximum conversion of 50 per cent of the protein into carbohydrate, the total yield of dextrose was 55 gm. Following ingestion of meat, the pyruvic acid level rose slowly (Table III), indicating that ingestion of protein may lead to an increase of keto acid.

Fat, on the other hand, did not apparently affect the pyruvic acid level, even when a large quantity was ingested (Table III).

TABLE II

Relation of Levels of Pyruvic and Lactic Acids and Sugar in Blood after Ingestion of Food

Time of blood collection	Experimental conditions	Subject E G *				Subject A†	
		Blood sugar	Pyruvic acid	Lactic acid	Lactic / Pyruvic	Blood sugar	Pyruvic acid
		mg per cent	*mg per cent*	*mg per cent*		*mg. per cent*	*mg. per cent*
12 noon	Lunch 12.05–12.30	77	0 69	8 5	12.3		
1 p.m.	Rested 12–1 p.m.; began work at 1 p.m.	112	1 91	21.3	11.1		
2 "	Laboratory or office work	73	0 99	12 9	13.0	95	0.48
3 "	" " " "	66	0.54	8 0	14.8	82	0.45
4 "	" " " "	80	0 72	9.8	13.6	91	0.48
5 "	" " " "	78	0 62	9 2	14.8	92	0.51
6 "	" " " "	71	0.63	8.2	13.0		

* The noon meal was the same as that in the previous experiment; see Table I.

† Subject A, an office worker, complained of feeling very tired. His breakfast consisted of orange juice, one egg, one slice of toasted bread and butter, and coffee. The noon lunch, which was taken in the laboratory, consisted of a sandwich and two cups of coffee with sugar.

Effect of Muscular Activity

Normal Activities Versus Rest—Results from three groups of subjects are shown in Tables IV and V. Group I consisted of laboratory and office workers who were carrying out their usual daily activities. Group II consisted of laboratory workers and medical students. The subjects of the latter group had no strenuous activities before they came to the

TABLE III

Effect of High Protein or High Fat Diet on Pyruvic Acid of Blood

The conditions with respect to work and rest were the same as shown in Table I, except that the subjects, four women laboratory workers, ate isocaloric quantities of meat or butter They rested from 12 noon to 1 p.m , after which they continued with their work. The results are expressed as mg. per cent.

Collection of blood samples	Meat of turkey, 850 calories				Butter, 850 calories			
	E S		G E. H		B B		E. B.	
	Pyruvic acid	Sugar	Pyruvic acid	Sugar	Pyruvic acid	Sugar	Pyruvic acid	Sugar
a m								
11.30	0.70	80	0.72	76		70	0.76	80
12	0 67	79	0 70	73	0 68	69	0 70	80
p m								
1	0.76	88	0.84	75	0.68	68	0.74	81
2	0.96	90	0 80	81	0.70	75	0.66	79
3	0.86	83	0 71	81	0 76	78	0.76	85
4	0.94	76	0.90	75	0.72	73	0.70	85
5	1.00	83	0.82	79	0.75	74	0.83	82
6	0.80	83	0.78	82	0.70	70	0.80	85

TABLE IV

Effect of Rest and Light Activity on Pyruvic Acid Level of Blood

Ethyl acetate solvent and Filter 520 were used. Samples were taken 3 to 5 hrs. after breakfast or the noon meal. The results are given in mg. per cent.

	Group I Usual activities of laboratory or office work	Group II After 0 5 hr. or more of rest in laboratory; no strenuous activities 2–4 hrs before coming to laboratory
No. of subjects	29	14
" " samples.	48	33
Pyruvic acid, maximum variation	0.44–0 93	0.60–0.95
" " arithmetic mean..	0.74*	0 77

* If all data from subjects E. G. and A (Tables I and II) are excluded, the maximum variations are from 0.54 to 0.93 and the arithmetic mean is 0.76.

laboratory. They rested 1 hour before the blood was drawn. Group III consisted of hospital patients, resting in bed. In every instance the blood was taken from 3 to 5 hours after the morning or noon meal.

The maximum variation of results is greater in Group I than that in Group II, and the average is slightly lower in Group I. However, if all data from subjects E. G. and A. of Group I (two from Table I and two from Table II) are excluded,[2] then the results fall within the limits 0.54 to 0.93, and the arithmetic mean is 0.76. The variations in Group II fall within the same range, 0.60 to 0.95, and the mean of all determinations is 0.77. It would seem, therefore, that the pyruvic acid level of the blood is not apparently increased after moderate activity.

Table V

Effect of Rest and Light Activity on Lactic-Pyruvic Acid Ratio

Groups I and II were the same as given in Table IV. Group III consisted of hospital patients who had rested from 1.5 to 4 hours in bed before samples were taken. This group includes only those patients whose pyruvic acid levels were within the range 0.60 to 0.95 mg. per cent.

	Group I 10 subjects		Group II 14 subjects		Group III. Hospital patients resting in bed, 16 subjects	
	Maximum variation	Arithmetic mean*	Maximum variation	Arithmetic mean*	Maximum variation	Arithmetic mean*
All results; samples taken hourly throughout the day	9.7–20.8	13.5				
3–5 hrs. after breakfast or noon meal	9.7–20.0	13.2	8.4–14.1	11.3†	9.6–20.0	13.4

* This is the arithmetic mean of the averages of all results from each subject.

† The averages of ratios obtained at various times from each of the fourteen subjects were 12.8, 10.3, 10.8, 10.0, 12.2, 13.0, 12.8, 12.4, 12.9, 10.3, 12 0, 12 2, 12.6, 10.0.

The magnitude and range of the ratio of lactic to pyruvic acid during work throughout the day are indicated in Tables I and II. Only three series of calculated ratios are shown; they are fairly representative of the entire group. The ratio is by no means constant throughout the day. The fluctuation from the mean is as great during the first 3 hours after a meal as in the subsequent hours. The ratio, therefore, is not materially affected by the usual rise and fall in the level of pyruvic acid following ingestion of food.

However, the range and magnitude of the lactic-pyruvic acid ratio are affected by activity (Table V). Thus, the results varied from 9.7 to 20.0 mg. per cent in Group I, and from 8.4 to 14.1 in Group II. The average in Group I was 13.2, as compared with 11.3 in Group II. Long rest in bed also appears to affect the ratio; in Group III, which consisted of sixteen hospital patients, the maximum range and arithmetic mean were almost identical with those of Group I. Since the range of the pyruvic acid level (see Tables IV and V) is the same in all groups, it is evident that the pyruvic acid level is not as readily affected as the level of lactic acid.

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[2] Subjects E. G. and A were the only subjects of forty-three studied whose blood attained unusually low levels of pyruvic acid.

TABLE VI

Effect of Walking or Running Distance of 1 Mile

July 29, 1941. The subject, J. H., ate breakfast at 6 a.m.

Time a.m		Comment	Time required for walk or run of 1 mile		Time of sampling after exercise		Lactic acid	Pyruvic acid	Lactic / Pyruvic
hrs	*min*		*min*	*sec*	*min*	*sec*	*mg per cent*	*mg per cent*	
9	30	Sample taken in laboratory after 0.5 hr of rest					10.9	0.83	13.0
9	50	Left the laboratory							
9	58	Arrived at track after walking about 200 yards							
10	3	Sample after 5 min. rest			5		11.5	0.81*	14.2
10	5	Began walking 1 mile slowly, rate 2.83 m.p.h. Rested 10 min.	21	10	5		10.7	0.72	14.9
10	37	Began walking 1 mile rapidly; rate 4.04 m.p.h. Rested 29 min.	14	52	5	50	10.2	0.83	12.3
11	21	Began jogging 1 mile, rate 6.48 m.p.h. Rested 10 min.	9	15	5	4	38.7	2.40	16.1
11	40	Began running 1 mile; rate 8.80 m.p.h.	6	49	6	2	139.5	4.98	28.0

Moderate Muscular Work—Owles (5) and Dill and coworkers (6, 7) have shown that moderate muscular work, such as a brisk walk, does not materially increase the level of lactic acid in the blood. Does it affect the pyruvic acid level?

The effect of walking a distance of 1 mile is shown in Table VI. The subject, an athlete, had been in training more than 2 months, running a distance of at least 4 miles each day. The tests were carried out on a hot, humid morning in July. As can be seen, a brisk walk at the rate of 4.04 miles per hour did not perceptibly increase either the lactic or pyruvic acid content, despite the fact that the subject perspired profusely. There was, in fact, a slight decrease in the lactic acid content of the blood after exercise. Such results have been observed in other experiments. Similar results were obtained by Owles (5).

At the same time after a walk of 1 mile at the rate of 3.93 miles per hour, the blood of subject A contained 0.86 and 10.7 mg. per cent of pyruvic and lactic acids, respectively. The blood before exercise contained 0.77 mg. per cent of pyruvic acid, and 10.4 mg. per cent of lactic acid. The lactic-pyruvic acid ratios before and after the walk were 13.6 and 12.5. Subject A, an office worker about 45 years of age, seldom exercised or indulged in sports.

Climbing stairs is considered a somewhat more strenuous form of exercise than walking. Subjects climbed two to three flights of steps at the rate of about one step per second. The height to which they climbed was calculated according to their weight The total work was about 650 kilogram-meters, which was accomplished in about 1 minute. The subjects then descended the steps at the rate of one step per second. The following typical results represent the pyruvic acid levels before and 7 minutes after the exercise: Mrs. C., 0.67, 0.85; Mrs. H., 0.82, 1.07; Mr. L., 0.79, 1.10; Mr. T., 0.75, 0.92.

Greater differences were observed after performance of approximately the same work by two elderly overweight women (Table VII). Their dietary history indicated a daily intake of less than 500 γ of thiamine over a period of several months prior to admission to the hospital. Their clinical signs and symptoms indicated a vitamin B complex deficiency. On admission they were given a reducing diet which was deficient in vitamin B complex and which contained from 350 to 500 γ of thiamine per day. The first test was made during the 1st week, the second 1 week later. As can be seen in Table VII, the resting pyruvic acid levels were within the normal range. The rise of pyruvic acid in the blood of Mrs. R. after administration of dextrose ((2), Table XIV) was quite normal; the thiamine tolerance was within the lower limit of normal.

Is the marked rise of pyruvic acid after work due to the vitamin deficiency, or does it reflect a low degree of physical fitness? The clinical observations favor the first view. However, the laboratory tests, which usually are regarded as reliable, were negative. To be noted is a further increased level of pyruvic acid after the second test. Does this indicate an increasing state of deficiency? If so, then tests which put stress upon the organism, such as a work test, should prove more sensitive for detecting incipient deficiency than the determination of pyruvic acid in the resting condition or after the ingestion of carbohydrate. It is possible, however, that the results reflect a low degree of physical fitness. These subjects took little exercise, perhaps less than at home. Furthermore, they were losing weight.

These experiments indicate the difficulties which may be encountered when work tests are applied to patients who are receiving inadequate diets. Several tests for thiamine deficiency, involving work, have been proposed (15–17). The amount of work to be done in such tests has not been adequately defined. It is doubtful whether it can be defined, since the reaction toward a given task is so greatly affected by the individual's physical state. This makes the interpretation of results difficult, and, therefore, limits the usefulness of work tests.

TABLE VII

Effect of Light Exercise; Hospital Patients

Subject	Experimental conditions	Time of sampling after exercise	Pyruvic acid	Lactic acid	Lactic acid / Pyruvic acid
		min	*mg. per cent*	*mg per cent*	
Mrs. McV.	4 hrs. of bed rest after breakfast	Before	1 08	13.3	12.3
" "	Climbed 2 flights of steps	8 0	1.70	21.5	12.6
" "	4 hrs. of bed rest after breakfast*	Before	0 88	13.5	15.3
" "	Climbed 2 flights of steps	7.0	2 11	28.3	13.4
" R.	4 hrs. of bed rest after breakfast	Before	0.71	14.2	20.0
" "	Climbed 2 flights of steps	7.0	1.48	26.4	17.8
" "	4 hrs. of bed rest after breakfast*	Before	0.85	15.0	17.7
" "	Climbed 2 flights of steps	7.0	1.68	26.4	15.7

* The second test was made 1 week later.

The high initial resting ratio of lactic to pyruvic acid should be noted (Table VII). Such results are observed when some subjects have rested a long period of time. This trend can be seen in the results from the selected group of sixteen patients shown in Table V. In such cases, the ratio was usually decreased by mild exercise. A similar trend toward lower lactic-pyruvic acid ratios was noted in subjects J. H. (Table VI) and A.

Effect of Simulated High Altitude[3]

The experiments were carried out in an insulated low pressure chamber of 460 cu. ft. capacity. An average temperature of 21.1° was maintained by a thermostatically controlled cooling system. Vibrations and noises were eliminated by appropriate damping devices. Reduction of pressure and return to the normal atmospheric pressure were at a rate corresponding to an ascent or descent of 1000 feet per minute. Oxygen was administered by means of a face mask provided with a rubber rebreathing bag, from which the oxygen was obtained.

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[3] The facilities for this work were kindly placed at our disposal by Dr. Andrew C. Ivy, to whom we are further indebted for many helpful suggestions. The effect of anoxia at high altitude will be discussed at length in a later paper.

Results of four experiments with one subject are given (Tables VIII to X) in order to show the variations in response to the same or new conditions in succeeding experiments. This subject appeared to be considerably more resistant to the effects of anoxia than other subjects used in similar experiments. Although severely affected in one of the experiments (see Table IX), his condition never resembled that of shock. On administration of oxygen his recovery was immediate and without after effects. This may account for the relatively normal ratios of lactic to pyruvic acid at 20,000 feet in Experiment 3 (Table IX) and the rapid return of the ratio to normal after administration of oxygen. It may account also for the relatively low ratio attained at high altitude in the other experiments.

TABLE VIII

Effect of 18,000 Feet of Altitude, with Short Exposures at Lower Levels; All without Oxygen

Subject D. W.; medical student, age 23 years.

Altitude	Experiment 1, Dec 1				Experiment 2, Dec 10			
	Time	Lactic acid	Pyruvic acid	Lactic/Pyruvic	Time	Lactic acid	Pyruvic acid	Lactic/Pyruvic
	p.m	*mg. per cent*	*mg. per cent*		*p.m*	*mg. per cent*	*mg. per cent*	
Ground level					3.50	8.6	0.75	11.5
" "	4.15	8.8	0.71	12.4	4.00	8.0	0.76	10.5
10 min. at 10,000 ft.	4.35	9.8	0.77	12.7	4.20		0.74	
10 " " 15,000 "	4.50	9.8	0.71	13.8	4.35*	8.2	0.76	10.8
10 " " 18,000 "	5.05†	12.7	0.82	15.5	4.50‡	11.9	0.71	16.8
20 min. at 18,000 ft.	5.15	12.8	0.84	15.2	5.00	13.5	0.71	19.0
30 " " 18,000 "	5.25	12.3	0.77	16.0	5.10	13.4	0.85	15.8
10 " " 10,000 "	5.45§	9.4	0.68	13.8	5.30‖	10.3	0.82	12.6
20 " " 10,000 "	5.55	9.6	0.67	14.3	5.40	12.0	0.80	15.0
5 " " ground level	6.10	9.8	0.67	14.6	5.55	10.3	0.84	12.3
15 " " " "	6.20	9.7	0.68	14.3	6.05	10.0	0.83	12.1

* Headache; slight cyanosis.

† Headache; markedly cyanotic.

‡ Marked cyanosis.

§ Cyanosis has disappeared.

‖ Cyanosis disappearing.

Another feature of these results is the apparent constancy of the pyruvic acid concentration in the blood at rest 4 hours or more after a meal, as determined in successive experiments. Thus, the initial concentrations at ground level were 0.71 mg. per cent, Experiment 1; 0.75, 0.76 mg. per cent, Experiment 2; 0.72 mg. per cent, Experiment 3; 0.66, 0.72 mg. per cent, Experiment 4.

These experiments show that the level of lactic acid is increased at high altitude. This is due to the anoxia, and not to altitude *per se*, since the rise of lactic acid is prevented by the administration of oxygen (Table X). On removing the oxygen mask at high altitude, the level of lactic acid was in most instances increased before any change was noted in the level of pyruvic acid. Thus, at a simulated altitude of 23,000 feet (Table X), 9 minutes after removing the mask, the lactic acid concentration rose from 8.3 to 10.7 mg. per cent, while the pyruvic acid concentration remained unaltered within the limits of error of the method. The rise of lactic acid was small; it was so small that it would otherwise be entirely overlooked, unless a procedure, such as was used here, with control periods at the beginning and end, was used. The rise occurred only at high

TABLE IX

Effect of 22,000 Feet of Altitude without Oxygen, Followed by Oxygen to the Ground Level

Subject D. W., Experiment 3, April 25. At about 5.10 p.m. the subject's attention was poor, but he apparently understood commands when repeated several times. He could not write and the sensation of pain was dulled. He had no memory of events from this time until after the oxygen mask was applied.

Altitude	Time		Oxygen	Lactic acid	Pyruvic acid	Lactic acid/Pyruvic acid
	Arrived at altitude	Sample taken				
ft	*p m*	*p m*		*mg. per cent*	*mg per cent*	
Ground level		3.55	None	7.9	0.72	11.0
15,000	4.19	4.29	"	8.4	0.72	11.7
18,000	4 31	4 41	"	9.7	0.78	12.4
18,000		4.51	"	10.7	0.90	11.9
20,000	4.53	5.03	"	12.4	1.03	12.0
22,000	5 06	5.16	"	16.1	1.04	15.5
22,000		5.23*	"	16.2	1.09	14.9
22,000		5 36	Oxygen	13.5	1.21	11.6
15,000	5 43	5.53	"	10.4	0.96	10.8
Ground level	6.09†	6.19	None	9.1	0.76	12.0
" "		6.29	"	9.6	0.73	13.1

* Oxygen was given immediately after this sample was taken.

† Oxygen was discontinued at this time.

altitude, and was abolished on the administration of oxygen (Table IX) or return to an altitude of 10,000 feet (Table VIII).

As already stated, the level of pyruvic acid was not as readily affected by anoxia as was that of lactic acid. Thus, no significant rise of pyruvic acid was seen after 10 minutes at 18,000 feet (Tables VIII and IX). However, *the concentration of both acids was increased at higher altitudes.*

The effect of oxygen on the relative concentration of lactic and pyruvic acids is shown in Tables IX and X. Thus, 13 minutes after administration of oxygen (Table IX), the level of lactic acid had *decreased* and the level of pyruvic acid had *increased*, resulting in a normal ratio. Again, at a concentration of 1.07 mg. per cent of pyruvic acid (Table X) the concentration of lactic acid after 41 minutes of anoxia at high altitude was 15.0 mg. per cent; it was 13.2 mg. per cent 15 minutes after oxygen was administered. The ratio in the first period without oxygen was 14.0, in the second with oxygen, 12.3.

TABLE X

Effect of 24,000 Feet of Altitude with Oxygen, without Oxygen, and Finally with Oxygen to Ground Level

Subject D. W., Experiment 4, May 18.

Altitude	Time				Oxygen			Lactic acid	Pyruvic acid	Lactic acid / Pyruvic acid
	Arrived at altitude		Sample taken							
ft	*hrs*	*min*	*hrs*	*min*		*hrs*	*min*	*mg per cent*	*mg per cent*	
Ground level			10	8				9.1	0.66	13.8
" "			10	20				8.2	0.72	11.4
23,000	10	55	11	5	On	10	35	8.3	0.70	11.9
23,000			11	15				9.6	0.76	12.6
23,000			11	26				8.3	0.80	10.4
23,000			11	36	Off	11	27	10.7	0.83	12.9
23,000			11	47				12.3	0.90	13.7
24,000	11	58	12	8				15.0	1.07	14.0
24,000			12	19	On	12	14	18.0	1.22	14.8
24,000			12	29				13.2	1.07	12.3
24,000			12	44				12.5	0.98	12.8
Ground level	12	58	1	9	Off at	12	58	10.1	0.74	13.6
" "			1	20				14.1	0.73	19.3

DISCUSSION

The concentration of pyruvic acid in the blood of resting subjects is relatively little affected by light physical exertion, by long rest in bed, or by short exposures to simulated altitudes up to 15,000 feet. Under these conditions, 3 to 5 hours after a meal, the concentration of pyruvic acid in the blood of forty-three normal healthy subjects varied from 0.54 to 0.96, with an average of 0.77 mg. per cent. These results are slightly lower than those given by other workers, although the maximum range is approximately the same. Thus, Kato and Li (18) consider a range of 0.60 to 1.0 mg. per cent as normal for infants and children. The latter determinations were made early in the morning, presumably before the children had ingested food. Blood was received into tared vessels which contained a measured volume of 10 per cent solution of trichloroacetic acid.

Wortis, Goodhart, and Bueding (19) noted that the level of pyruvic acid in children was the same as that in adults. Thus the blood of thirty-five children contained from 0.71 to 1.21 with an average of 0.96 mg. per cent, and that of 60 adults, from 0.77 to 1.16, with an average of 0.98 mg. per cent. Their samples were obtained from fasting subjects at rest in bed, the samples being stabilized with fluoride and iodoacetate. As shown in a previous paper (1), blood which is stabilized by means of iodoacetate yields slightly higher results than the freshly drawn untreated samples. The differences vary from 0.05 to 0.20 mg. per cent and are not due to loss of pyruvic acid in the untreated blood, since the concentration in the untreated blood does not change within 3 minutes after collection. If a correction of 0.10 is applied to the data of Wortis, Goodhart, and Bueding, then their results from adults vary from 0.67 to 1.06, the average being 0.88 mg. per cent. By using the methods of Bueding and coworkers, Klein (20) observed a mean concentration of 0.76 ± 0.173 (mean deviation) mg. of pyruvic acid in the blood of twenty-four normal subjects under basal conditions. Our data, therefore, are in substantial agreement with those which have been obtained under basal conditions.

On the other hand, the resting, or basal level of lactic acid and its relation to pyruvic acid are not so readily stated, since they are affected by conditions which tend to produce tissue anoxia. In the opinion of Stotz and Bessey (16), the relation of lactate to pyruvate is constant under a wide variety of conditions, the ratio being 12.2 of lactate to 1 of pyruvate. We have never observed such a fixed relation. Under the prescribed conditions, the ratios varied from 8.4 to 14.1, with an average of 11.3. The average concentration of lactic acid was 8.7 mg. per cent. Bueding and Goldfarb (21) noted an average of 7.0 mg. per cent of lactic acid in the blood of fasting subjects at rest in bed, the samples having been collected without stasis and stabilized by means of fluoride and iodoacetate.

Since the principal concern of this paper is in technique, we shall discuss briefly some possible applications of the lactic-pyruvic acid ratio. In 1941, Friedemann and Barborka (22) called attention to the use of this ratio as a means of determining the relative oxidative conditions in the body. In a later paper the use of the β-hydroxybutyrate-acetoacetate ratio was suggested (23). This was based (*a*) on theoretical considerations, (*b*) on data obtained after strenuous muscular exercise, and (*c*) on the results which were presented in this paper. All tissues, as far as is known, contain lactate oxidase and diphosphopyridine nucleotide, both of which are necessary in attainment of the equilibrium between lactate and pyruvate.[4] The analysis of blood coming from a small area of tissue should, therefore, yield differences in the ratio in accordance with the supply of oxygen, provided that (*a*) approximate equilibrium is obtained between blood and tissue, (*b*) the two forms diffuse out of the tissue at the same rate, and, most important of all, provided that (*c*) the ratio is not changed rapidly in the blood. Both ions diffuse rapidly, since the concentration of both acids reaches the maximum within a few minutes after severe exercise (22) and since the maximum conversion of one acid into the other is obtained within 10 to 15 minutes after parenteral administration (14). Friedemann and Haugen (1) have shown that the concentration of lactic and pyruvic acids is not changed perceptibly in blood within 3 minutes after collection. Therefore, insofar as points (*b*) and (*c*) are concerned, the ratio of the acids in the blood probably approximates that in the tissue. The most serious objection to our use of the lactic-pyruvic acid ratio as an indicator of the oxidative state in tissues is the fact that we have analyzed blood from the arm veins. The concentration of substances in blood from the arm veins is the result of changes not only in the hand and forearm but also to some extent in other tissues. Since this was used throughout the study, the results should reflect the effect of various conditions not only upon the equilibria in the tissues of the arm, but also elsewhere in the body. The two acids are constantly being produced by tissues, some producing more than others, and some retaining the acids and storing them as glycogen (25). However, the ratio of the two acids in venous blood from the various tissues should be approximately the same, since all contain the necessary enzymes for establishing the equilibrium and since the blood supply to tissues and organs is usually sufficient for normal function. It is possible, therefore, that the ratio of lactate to pyruvate is approximately the same in all tissues of the normal resting subject, although the rate of production and removal of the acids may differ greatly in the various tissues.

Whether or not one may agree with these conclusions, our results demonstrate that the oxidative condition in the body as a whole, as judged by the lactate-pyruvate ratio, is regulated with considerable constancy within a wide range of activity of the normal subject. Thus, the ratio is not materially altered in the same subject whether he is resting or performing light muscular exercise, such as walking (see Table VI).

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[4] For a discussion of the mechanism of the reaction and the pertinent literature, see the review by Barron (24).

That the determination of the ratio may be of value in problems dealing with circulatory insufficiency or failure and anoxia at high altitude is indicated by the results which were presented in this paper: (*a*) the ratio as well as the absolute concentration of pyruvate and lactate is unchanged at high altitude when subjects breathe oxygen; (*b*) the ratio is increased at simulated altitudes above 15,000 feet, and (*c*) is rapidly brought within the normal range on administration of oxygen or by return to an altitude of 10,000 feet; (*d*) the ratio in some subjects may be increased by long inactivity, and (*e*) if elevated, it may be decreased by mild muscular exercise. Since the anoxia at high altitude is general throughout the tissues, our data indicate the degree of anoxia which is necessary to bring about a perceptible change in the ratio. Point (*d*) indicates that at times the degree of anoxia due to local stagnation of blood is as great as that which is obtained at high altitudes, point (*c*) indicates the beneficial effect of activity, which has long been recognized.

The older literature concerning the effect of high altitude contains many references to "acidosis" which is attributed to the accumulation of large quantities of lactic acid in the blood. These statements are based upon measurements of alveolar CO_2 tension and determinations of lactic acid, the latter by relatively crude methods.[5] However, by using more precise methods, Edwards (28) found that the fasting resting level of lactic acid was not increased in *acclimated* subjects. Our data beyond question demonstrate not only that the lactic acid level but also the pyruvic acid level is increased in *unacclimated* subjects. The changes are small at low altitudes, and, without the use of control periods at the beginning and end of the experiments, may well be overlooked. They occur in two stages: (*a*) a slight increase of lactic acid, without change of pyruvate, which is noted at altitudes of 15,000 to 18,000 feet, and (*b*) a marked increase of both acids at altitudes above 18,000 feet. It should be noted that the first change occurs at the range of altitudes at which the physical and mental performance of human subjects is progressively decreased (26, 27). The second series of changes occurs at altitudes at which most subjects soon lose consciousness. The often dramatic effect of anoxia is attributed to functional impairment of the nervous system. Our results indicate that the anoxia at altitudes greater than 18,000 feet depresses the oxidative processes in other tissues throughout the body to such an extent that the rate of metabolism of pyruvic acid no longer keeps pace with the breakdown of sugar, in consequence of which the energy needs cannot be met by oxidation.

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[5] For a review of the literature, see the monographs by Armstrong (26) and Van Liere (27).

SUMMARY

Experiments were described showing the effect of ingestion of food, light muscular activity, and anoxia on the concentration of pyruvic and lactic acids in the blood. These three conditions probably affect the levels of the acids most in the normal adequately nourished subject. On the basis of these experiments, a procedure for taking blood was adopted which should be useful in physiological experiments and in the clinic where it may be impossible, or even undesirable, to take blood from subjects under basal conditions.

Ten office and laboratory workers received breakfast and lunch in the laboratory, after which they performed their usual work. The concentration of both acids was increased and reached the maximum 1 to 2 hours after the meal; it returned to the original level within 3 to 4 hours. The level rose slightly after ingestion of meat equivalent to 850 calories; no effect was noted after ingestion of an isocaloric quantity of butter. Forty-eight samples of blood from these and nineteen other subjects, while at work 3 to 5 hours after the meal, contained from 0.44 to 0.93, with an average of 0.74 mg. per cent of pyruvic acid. Thirty-three samples, which were taken 3 to 5 hours after a meal from fourteen normal subjects after they had rested 0.5 hour or more, contained from 0.60 to 0.95, with an average of 0.77 mg. per cent of pyruvic acid. The usual activities of office or laboratory work, therefore, did not lead to a measurable elevation of the pyruvic acid content of the blood. The results were within the same limits as those obtained by other investigators from fasting resting subjects.

Light muscular exercise, such as a brisk walk, did not measurably affect the lactic and pyruvic acid levels. It is evident that the energy for such activity can be obtained entirely by oxidation, without accumulation of either pyruvic or lactic acid in the blood.

However, the concentration of both acids was increased slightly after subjects had climbed steps; the work was equivalent to 650 kilogrammeters, which was performed in 1 minute. Greater differences were obtained after the same work by two elderly overweight women who were receiving a reducing diet which was deficient with respect to vitamins of the B complex. The application of work tests as a means of detecting thiamine deficiency was discussed briefly.

The rise of the lactic-pyruvic acid ratio as a means of detecting the effect of anoxia was discussed. The oxidative conditions in the body as a whole, as judged by the ratio, are regulated with considerable constancy

within a wide range of activity of the normal subject. The ratio may be increased as the result of long inactivity; if elevated, it may be decreased by light muscular exercise.

Results from four experiments with one subject at simulated altitudes up to 24,000 feet were presented in order to demonstrate the application of the technique to a problem of great present interest. (*a*) The lactic acid content of the blood was increased slightly at altitudes of 15,000 to 18,000 feet, the pyruvic acid content remaining unchanged; (*b*) the levels of both acids were increased at altitudes above 18,000 feet. These changes were prevented by breathing oxygen; when the levels were elevated due to the anoxia, they were rapidly reduced to within the "normal" limits by breathing oxygen or by return of the subjects to a simulated altitude of 10,000 feet.

BIBLIOGRAPHY

1. Friedemann, T. E., and Haugen, G E., *J. Biol. Chem.*, **144**, 67 (1942).
2 Friedemann, T E., and Haugen, G. E , *J. Biol. Chem.*, **147**, 415 (1943).
3 Friedemann, T. E., Cotonio, M., and Shaffer, P. A , *J. Biol. Chem.*, **73**, 335 (1927). Friedemann, T. E , and Graeser, J B., *J. Biol Chem.*, **100**, 291 (1933).
4. Barr, D P., Himwich, H. E., and Green, R P., *J. Biol. Chem.*, **55**, 495 (1923).
5. Owles, W. H , *J Physiol* , **69**, 214 (1930).
6 Talbott, J H , Folling, A., Henderson, L. J., Dill, D. B., Edwards, H. T., and Berggren, R. E L., *J Biol. Chem.*, **78**, 445 (1928).
7 Margaria, R., Edwards, H T., and Dill, D. B., *Am. J. Physiol.*, **106**, 689 (1933).
8. Trimble, H. C., and Maddock, S. J., *J. Biol. Chem.*, **81**, 595 (1929).
9. Haggard, H. W., and Greenberg, L. A., Diet and physical efficiency, New Haven (1923)
10. Lu, G. D , *Biochem. J.*, **33**, 249, 774 (1939).
11. Somogyi, M., *J. Biol. Chem.*, **86**, 655 (1930).
12. Shaffer, P A , and Hartmann, A. F., *J. Biol. Chem.*, **45**, 365 (1920–21). Shaffer, P. A., and Somogyi, M , *J. Biol. Chem.*, **100**, 695 (1933).
13. Bueding, E , Stein, M. H., and Wortis, H , *J. Biol. Chem.*, **140**, 697 (1941).
14 Bueding, E , and Goldfarb, W , *J. Biol Chem* , **147**, 33 (1943).
15. Lu, G. D., and Platt, B. S., *Biochem. J.*, **33**, 1538 (1939).
16 Stotz, E., and Bessey, O. A , *J Biol. Chem* , **143**, 625 (1942).
17. Williams, R. D , and Kendall, E. C., *Arch. Int. Med.*, **72**, 185 (1943).
18 Kato, K., and Li, P , *Am. J. Dis. Child.*, **61**, 1222 (1941).
19. Wortis, H , Goodhart, R , and Bueding, E., *Am. J. Dis Child* , **61**, 226 (1941).
20. Klein, D., *J. Biol Chem* , **145**, 35 (1942).
21. Bueding, E., and Goldfarb, W., *J. Biol. Chem.*, **141**, 539 (1941).
22. Friedemann, T. E , and Barborka, C. J., *J. Biol Chem.*, **141**, 993 (1941).
23. Friedemann, T. E., *J. Biol. Chem.*, **142**, 635 (1942).
24. Barron, E. S. G., *Physiol. Rev.*, **19**, 184 (1939).
25. Himwich, H. E., Koskoff, Y. D., and Nahum, L. H., *J. Biol. Chem.*, **85**, 571 (1929–30).
26. Armstrong, H. G., Principles of aviation medicine, 2nd edition, Baltimore (1943).
27. Van Liere, E. J., Anoxia, its effect on the body, Chicago (1942).
28. Edwards, H. T., *Am. J. Physiol.*, **116**, 367 (1936).

A NOTE ON THE PREPARATION OF CARDIOLIPIN

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Recently an improved method for the preparation of cardiolipin was described[1] which employed precipitation with $BaCl_2$ from methanol extracts of beef heart and subsequent treatment with NaCl to convert barium salts to sodium salts. It was reported that there was little loss of cardiolipin into the fraction insoluble in methanol which consisted largely of unchanged barium salts. This statement requires modification in the light of later experience.

The use of NaCl for the decomposition of the crude barium salts was based on its uniformly successful use with pure or nearly pure salts of cardiolipin. In relatively small scale preparations (five hearts), this method was successfully applied to the recovery of cardiolipin from crude mixtures. In later preparations on a larger scale, however, it proved difficult to obtain consistently good yields. In the presence of large amounts of impurities such as are found in the original crude barium precipitate, the NaCl method appears to be insufficient for the complete recovery of cardiolipin and should be replaced or supplemented by the use of Na_2SO_4, as illustrated by the following typical experiment.

The crude barium precipitate from the first methanol extract of 13 kilos of tissue (wet weight) was fractionated as previously described[1] after treatment with NaCl. The fraction insoluble in methanol was collected by centrifugation and the bulky precipitate stirred up in the centrifuge cup with twice its volume of ether and about 40 ml. of 5 per cent Na_2SO_4. The precipitate gradually dissolved in the ether layer while $BaSO_4$ separated and was removed by centrifugation. The ether layer was transferred to a separatory funnel and shaken vigorously with three successive portions of 5 per cent Na_2SO_4. It was noticeable that the ether layer persistently retained $BaSO_4$ and insoluble organic impurities in a colloidal state. In order to obtain a clear ethereal solution it was necessary to add alcohol, 2 or 3 ml. at a time, until the insoluble matter flocculated and could be centrifuged out. The ethereal solution was then dried on Na_2SO_4, filtered, concentrated, and poured into methanol; the precipitate which separated was thoroughly washed with methanol and discarded. The methanol solution, containing 10.5 gm. of material, was precipitated with 20 per cent aqueous $BaCl_2$. The precipitate was washed successively with methanol and acetone and reprecipitated several times from ether by an equal volume of acetone.[1] It formed gelatinous solutions in ether, characteristic of cardiolipin. The purified barium salt was converted to the sodium salt by shaking with half saturated NaCl and yielded 6.4 gm. of sodium cardiolipin.

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[1] Pangborn, M. C., *J. Biol. Chem.*, **153**, 343 (1944).

In a similar experiment with another lot of tissue, only a small yield of barium cardiolipin was obtained, most of the barium salt being soluble in equal parts of ether and acetone. The ether-acetone solution was concentrated by distillation until most of the ether was removed, then chilled in ice. A gummy precipitate separated. This was dissolved in ether and decomposed with 5 per cent Na_2SO_4 as described above. The ethereal solution was dried, filtered, concentrated, and poured into absolute alcohol. The precipitate was treated with several portions of methanol, and a small insoluble residue discarded. The methanol-soluble material so obtained was purified over the barium salt as described above and proved to consist largely of cardiolipin.

Experience indicates that for maximum yields of cardiolipin all ether-acetone supernatants from reprecipitation of the barium salts should be repurified at least once in this manner. The larger the proportion of impurities in the mixture, the greater will be the loss of barium cardiolipin into the ether-acetone solutions.

RIBOFLAVIN IN BLOOD PLASMA OF SOME BRAZILIAN SNAKES

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Blood plasma from snakes of the Brazilian poisonous *Bothrops* and the non-poisonous *Eudryas* is yellow-green in color and when observed through ultraviolet light (mercury lamp with Wood's filter) presents an intense green-blue fluorescence. This observation has suggested to us a detailed study of the plasma pigments of some snakes. When the blood plasma proteins are precipitated with 50 per cent methanol and dilute acetic acid in a hot water bath, the fluorescence of the filtrate is strongly intensified. The extraction of the methanol-water solution with light petroleum ether permits the separation of the yellow-green fluorescence from the blue. Some experiments were executed to identify this greenish fluorescence with riboflavin. Thus the fluorescence disappears by reduction and reappears by oxidation in air. Chloroform and ether do not extract the pigment. Dialysis experiments with cellophane bags demonstrated that only a small percentage of the flavin is in the free state. The flavin is adsorbed with lead sulfide and fullers' earth and eluted by pyridine-water-acetic acid mixture as in the case of riboflavin. Lumiflavin was obtained by irradiation of the watery extract after protein precipitation by heat. Potassium permanganate was ineffective in reducing the green fluorescence. The blue-violet fluorescence which is extracted by petroleum ether is resistant to hydrosulfite and probably belongs to the unknown blue fluorescent compound obtained by von Euler and Adler from the retina of fishes (1).

On the other hand the blood plasma of the rattlesnake (*Crotalus terrificus*) and that of the *boipeva* (*Xenodon merremii*) are practically devoid of flavin and the deproteinized extracts exhibit a blue-violet fluorescence. It is interesting to recall that Taborda and Taborda showed in 1941 that the venom of some species of *Bothrops* is rich in riboflavin and that of the rattlesnake presents only a bluish fluorescence (2).

EXPERIMENTAL

Blood plasma was collected from decapitated snakes in tubes with 0.3 per cent potassium oxalate to prevent coagulation. The last portions of the bleeding were discarded to avoid contamination with tissue fluids. After thorough mixing, the blood was centrifuged and clear, non-hemolyzed plasma was pipetted off. Extraction was usually performed on the same day that the blood was withdrawn. Some samples, however, were stored in the ice box under sterile conditions and well protected from light. The present report is based on experiments with 52 snakes (thirty *Bothrops jararaca*, ten *Xenodon merremii*, nine *Crotalus terrificus*, and four *Eudryas bifossatus*).

Extracts were prepared as follows: To 2 ml. of clear plasma, 10 ml. of 50 per cent methanol and 0.25 ml. of 0.15 M acetic acid are added in a Pyrex test-tube. This tube is kept in a boiling water bath for 2 to 3 minutes. After cooling, the liquid is filtered. The clear filtrate is slightly yellowish. The precipitate left in the filter is washed with 2 ml. of water. The final volume of the filtrate is adjusted to 12 ml. This solution is extracted with 4 ml. of petroleum ether to separate the blue-violet fluorescent compound. The extract free from petroleum ether is placed in the cup of the Pfalz and Bauer fluorophotometer and the galvanometer readings are recorded. To the same filtrate, 0.3 to 0.5 gm. of sodium hydrosulfite is added and mixed thoroughly with a glass rod. Air bubbles must be avoided. The disappearance of the fluorescence is immediately evident. The solution is then placed in a well stoppered test-tube, vigorously agitated and again the fluorescence recorded in the fluorometer.

TABLE I

Reduction of Flavin in Plasma Extracts

Plasma extracts	Galvanometric units				
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
Bothrops jararaca	20	24	22	16	
" " + hydrosulfite	4	3	2	3	
Oxidation by air	15	20	20	14	
Eudryas bifossatus	28	26	27	28	32
" " + hydrosulfite	6	3	2	4	2
Oxidation by air	20	18	22	24	28

These results are summarized in Table I.

Other experiments were conducted to determine the flavin content of plasma. The filtrate obtained as stated above was treated with lead sulfide which specifically adsorbs the flavin (3). To a Jena sintered glass funnel (G3) containing a wet layer of freshly prepared lead sulfide as recommended by Emmerie, 10 ml. of the filtrate are added and thoroughly mixed with a thin glass rod. After 2 minutes the liquid is filtered with suction and the adsorbate is washed with 2 ml. of distilled water. The filtrate is devoid of flavin (see Table II). To the lead sulfide 10 ml. of the pyridine-water-acetic acid mixture (3:7:2) are added. The suspension is filtered and the lead sulfide is washed once more with the same mixture. The eluate was found to contain all the flavin of the original plasma.

Experiments with fullers' earth showed that the flavin is adsorbed from the methanol extracts.

It was not possible to extract the pigment with chloroform from the methanol or the watery deproteinized extracts. However, when these extracts were alkalinized with sodium hydroxide to pH 8.8 and exposed to illumination of a 250 watt lamp for 4 to 6 hours at 20°, it was possible to obtain a chloroform extract showing a yellow-green fluorescence (lumiflavin). The addition of 2 per cent potassium permanganate to the pyridine eluate did not appreciably change the intensity of the green fluorescence (±2 galvanometric units).

TABLE II

Adsorption Experiments with Lead Sulfide

Plasma	Color in daylight	Fluorescence in ultraviolet light			
		Plasma	Methanol extract	Filtrate not adsorbed	Eluate
*Bothrops jararaca**	Green	Bluish green	Green	Light blue	Green
*Crotalus terrificus**	Yellowish	Bluish	Blue violet	Blue violet	No fluorescence
*Xenodon merremii**	Light yellow	Light blue	Light blue	Light blue	Light green
Eudryas bifossatus	Green	Bluish green	Green	Blue	Green

* Pooled plasma from six snakes.

Fluorometric Determination—Quantitative determinations were carried out preferably with the pyridine eluate. The final volume of the eluate corresponding to 2 ml. of plasma was adjusted to 12 ml., which is the capacity of the cup of the fluorophotometer. In some cases 5 ml. of plasma were employed.

The Pfalz and Bauer apparatus, model A, equipped with special filters for riboflavin (peak between 4300 and 4400 A) was used. All readings were at maximal intensity of fluorescence. Riboflavin standard solutions containing 0.1 to 2 γ per 10 ml. were prepared daily from a stock solution, 10 mg. per 100 ml., kept in an ice box and well protected from light. The dilution was accomplished with pyridine-water-acetic acid mixture, as was done for the plasma eluate samples

Table III shows comparative results obtained by the fluorometric and microbiological methods.

Microbiological Assays—To ascertain the biological activity of the flavin present in the plasma some quantitative determinations were performed with the Snell and Strong microbiological method with *Lactobacillus casei* ε as the test organism (4).

Diluted blood plasma and deproteinized water extracts were employed. Whole plasma diluted with the basal medium gave somewhat higher values and therefore was discarded. This stimulatory effect is probably due to some factor present in blood, as observed by Feeney and Strong (5). The plasma filtrate extracted by petroleum ether lost this stimulatory effect. The deproteinized filtrate was prepared as follows: 4 ml. of plasma were diluted with 4 ml. of 1 per cent acetic acid, heated in a boiling water bath for 2 minutes, and passed through Whatman No. 42 filter paper. The clear filtrate is extracted with 4 ml. of petroleum ether and the aqueous layer used for the test. A series of twelve tubes was prepared for each determination. Volumes of 0.05 to 0.50 ml. with 0.05 ml. intervals were added to the basal medium. The control tubes were prepared with 0.05 and 0.1 ml. of photolyzed plasma filtrates. The standard curves were established with pure riboflavin (Hoffmann-La Roche), as recommended by Snell and Strong (4).

TABLE III

Comparison of Results Obtained by Fluorometric and Microbiological Methods

The results are expressed in micrograms per 100 ml. of plasma.

Method	*Bothrops jararaca*				*Eudryas bifossatus*			
Fluorometric	162	210	200	218	190	310	318	288
Microbiological	160	212	205	210	191	302	302	290

Recovery experiments were also performed by adding 0.1 γ of riboflavin to each of the serial tubes containing the same quantities of the deproteinized filtrate. Recoveries from 95 to 108 per cent have been observed. With the whole diluted plasma the recoveries were above 100 per cent. Good agreement was obtained between the determinations secured by the fluorometric method and by the Snell and Strong microbiological method (see Table III). Precautions were taken to avoid direct light during the determinations. Some operations (filtration, adsorption) were performed in the dark room.

Carotenoids—The presence of carotenoid pigments was observed in the plasma of *Bothrops jararaca* and *Eudryas bifossatus*. Only xanthophyll was encountered. No carotene or xanthophyll could be detected in the plasma of the genera *Crotalus* and *Xenodon*.

2 ml. of plasma were precipitated with 50 per cent methanol and dilute acetic acid in a boiling water bath. The yellowish protein precipitate was extracted with ethyl ether (4 ml.) which assumed an intense yellow color. Petroleum ether does not extract this pigment. With the Pulfrich step-photometer a maximum of 4300 A was found. The reaction of Haagen-Smit, Jeffreys, and Kirchner with phosphoric acid for xanthophyll was positive (blue color) (6). The plasma when treated by the technique of Clausen and McCoord for carotenoids revealed only xanthophyll (7). Chromatographic analyses made on adsorption tubes of 10 mm. diameter filled with calcium carbonate and aluminum oxide, as described by Zechmeister and Cholnoky (8), showed that the pigment is retained in the calcium carbonate layer.

SUMMARY

Blood plasma from snakes of the species *Bothrops jararaca* and *Eudryas bifossatus* contain riboflavin averaging 200 γ per 100 ml.

The plasma from the rattlesnake (*Crotalus terrificus*) and *Xenodon merremii* is practically devoid of flavins. The analytical study and the quantitative determinations were performed by fluorometric and microbiological methods. Xanthophyll was present in the plasma of the former species. Carotene was absent.

BIBLIOGRAPHY

1 von Euler, H , and Adler, E , *Z physiol Chem* , **228**, 1 (1934)
2 Taborda, A , and Taborda, L C., *Mem Inst Butantan*, **15**, 47 (1941)
3 Emmerie, A , *Acta brev Neerl* , **6**, 1 (1936)
4 Snell, E E , and Strong, F M , *Ind and Eng Chem* , *Anal Ed* , **11**, 346 (1939).
5 Feeney, R E , and Strong, F M , *J Biol Chem* , **142**, 961 (1942)
6 Haagen-Smit, A J , Jeffreys, C P , and Kirchner, J G., *Ind and Eng Chem* , *Anal Ed* , **15**, 179 (1943)
7 Clausen, S W , and McCoord, A B , *J Biol Chem* , **113**, 89 (1936)
8 Zechmeister, L , and Cholnoky, L , Principles and practice of chromatography, translated by Bacharach, A L , and Robinson, F A , New York (1941).

ON THE UTILIZATION OF RAFFINOSE BY PSEUDOMONAS SACCHAROPHILA

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It has previously been reported (1) that the bacterium *Pseudomonas saccharophila* is capable of utilizing the trisaccharide raffinose at a greater rate than any of the constituent hexoses, provided the organism is "adapted" to this compound by cultivation in a medium containing raffinose as the only carbon source. Similarly, cultures adapted to sucrose, trehalose, and maltose, respectively, can use these sugars more rapidly than their monosaccharide components. The recent demonstration of a reversible phosphorolysis of sucrose by this organism (2–5) has shed some light on the mechanism of the breakdown of this sugar, but, as already pointed out (2), has failed to explain the high rates of utilization of other disaccharides. It has been mentioned that dry cell preparations of bacteria grown with dissacharides other than sucrose (*e.g.* trehalose) as substrates showed no phosphorylase activity either on these sugars or on sucrose. Since raffinose is structurally related to sucrose, it seemed of interest to investigate its utilization by *Pseudomonas saccharophila*, especially when it was found that dry cell preparations made from cultures grown with raffinose as substrate would cause a rapid esterification of inorganic phosphate when either raffinose or sucrose was added. Since partially purified sucrose-phosphorylase had been shown to be inactive towards raffinose (3), it was first necessary to test the possibility of finding a new phosphorolytic enzyme.

The experiments presented here include (1) a study of the respiratory utilization of raffinose and its component sugars by suspension of intact resting cells, (2) an investigation of the hydrolytic and phosphorolytic reactions catalyzed by dry cell preparations of bacteria grown with raffinose as substrate, and (3) the demonstration of the occurrence of phosphorolytic reactions *in vivo* by the extraction of phosphoric esters from whole cells.

Oxidation of Substrates by Intact Cells—The bacteria were grown in a medium like that previously described (2) except that raffinose was substituted for sucrose as substrate. The cells were harvested by centrifugation, washed, and "starved" by continuous aeration in phosphate buffer at pH 6.64 for 18 hours at 30°. They were again concentrated and resuspended in buffer. Various substrates were then added to the suspension in Warburg respirometer vessels, and the rate of oxygen consumption measured at 30°. Since raffinose is composed of galactose, glucose, and fructose in such a way that it can be hydrolyzed either to melibiose and fructose or to galactose and sucrose, the oxidation of these disaccharides alone and with the hexoses was investigated. The Q_{O_2} values are given in Table I.

It will be noted that raffinose, melibiose, and sucrose could all be oxidized more rapidly than the constituent hexoses alone or in combination. It had already been shown (1) that in such experiments as this sugars are utilized with approximately two-thirds assimilation and one-third oxidation to CO_2. Approximately the same fraction of raffinose and of sucrose was found to be assimilated in the present studies. The rate of oxygen uptake may therefore be taken as a fair measure of the rate of sugar breakdown. However, a striking difference in the dependence of the rate of utilization on the concentration of various substrates is evident from Table I. Thus, a 40-fold increase in the concentration of raffinose or sucrose causes less than a 2-fold increase in respiration, while a similar change in the concentration of melibiose results in at least a 6-fold, and more likely a 20-fold increase in the rate of its utilization (depending on whether autogenous respiration continues during the slow oxidation of this sugar in low concentration (1)). A similar phenomenon has been previously reported for the utilization of mannose (1). That this was not due to the presence of rapidly oxidizable impurities in the concentrated melibiose was shown by the fact that the high rate continued unabated after over 5 per cent of the theoretical amount of oxygen required for the complete oxidation of the added melibiose had been consumed. Similarly, the relatively high rate observed with M/200 melibiose continued after more than 10 per cent of the sugar had been oxidized.

Investigation of Extracellular Enzymes—Since the first steps in the breakdown of raffinose might involve the hydrolysis of the trisaccharide by an extracellular enzyme or enzymes, as has been shown to occur in the utilization of glycogen and starch by the same organism (1), a search was made for hydrolytic enzymes in the medium after bacterial growth had taken place. Raffinose may be hydrolyzed to melibiose and fructose by the action of invertase, or to galactose and sucrose by melibiase.

Raffinose, sucrose, and melibiose were added in M/20 concentration to aliquots of the culture medium freed of bacteria by centrifugation and the mixtures incubated under toluene at 30°. The extent of hydrolysis of each sugar could be computed from changes in reducing value of the solutions. Only insignificant traces of hydrolytic enzymes could be detected in the medium (less than 0.1 mg. of any of the sugars decomposed per ml. in 24 hours at 30°).

In addition, no phosphorolytic enzymes were found to occur extracellularly, as was shown by the absence of any esterification of inorganic phosphate in the culture medium.

Experiments with Dry Cell Preparations—Dry cell preparations of *Pseudomonas saccharophila* were made as previously described (2), by the method originally suggested by Dr. Fritz Lipmann. A rapid esterification of inorganic phosphate was observed when such preparations were allowed to act on sucrose or raffinose in phosphate buffer. That the phosphate uptake was due to a phosphorolytic reaction was evident from the fact that it was not accompanied by respiratory or fermentative activity and that it was not inhibited by fluoride. Although it appeared most likely that the same phosphorylase is involved in this reaction as in the previously studied breakdown of sucrose, it was also possible that a direct phosphorolysis of raffinose or of melibiose might occur.

TABLE I

Utilization of Sugars by Intact Cells

Rates of oxygen utilization expressed as Q_{O_2} found with washed starved cells of *Pseudomonas saccharophila*, grown with raffinose as sole carbon source, and allowed to oxidize various substrates at 30° in Warburg respirometer vessels.

Substrate	Q_{O_2} with various substrate concentrations		
	M/800	M/200	M/20
None (autorespiration)	8.8	8.8	8.8
Raffinose	41.9		76.5
Sucrose	41.5		68.9
" + galactose	46.3		80.0
Melibiose	12.8	24.1	80.5
" + fructose	13.0		87.5
Glucose	12.0		16.0
Fructose	8.6		11.4
Galactose	12.8		22.6
Glucose + fructose + galactose	15.0		29.9

The dry cell preparations, unlike the culture medium, showed both melibiase and invertase as well as phosphorylase activity. The melibiase activity was measured by changes in reducing value, as well as by polarimetric means with melibiose as substrate. Determinations made by the two methods were in good agreement. The extent of inversion of sucrose was computed from the amount of reducing sugar produced in excess of phosphate used for the phosphorolytic reaction, as explained in an earlier paper (3). Although a strong hydrolytic action on raffinose was evidenced by the production of reducing sugar far in excess of phosphate consumption, no attempt was made to express it quantitatively, since the ratio of the initial, intermediate, and final products (a total of seven compounds) could not be conveniently estimated during the course of raffinose breakdown. The phosphorolytic and hydrolytic activities of the dry cell suspensions are shown in the first two columns of Table II. It will be seen from Table II that the initial rate of phosphate uptake was greater with sucrose than with raffinose as substrate, and that no esterification whatever occurred with melibiose. The hydrolytic action on melibiose, on the other hand, was, under the conditions of the experiment, greater than the combined phosphorolytic and hydrolytic decompositions of sucrose.

To determine whether any direct phosphorolytic breakdown of raffinose occurs or whether the phosphate uptake is due entirely to the phosphorolysis of sucrose produced from raffinose by hydrolysis, the phosphoric ester formed during the decomposition of raffinose by the dry cell preparation was isolated. This was found to be almost exclusively glucose-1-phosphate, which was identified by the methods previously described (2).[1] That no appreciable amounts of phosphoric esters of fructose, galactose, or melibiose are formed in the reaction was shown by the negative results of tests for ketoses (Roe's method) and for galactose (oxidation with nitric acid) on the precipitated barium salts. This indicated that raffinose does not undergo a phosphorolytic cleavage.

TABLE II

Phosphorolysis and Hydrolysis of Sugars by Preparations of Dry Cells of Pseudomonas saccharophila Grown with Raffinose

The preparations were allowed to act on M/10 melibiose, M/10 sucrose, and M/10 raffinose, respectively, in M/40 phosphate buffer at pH 6.64 for 10 minutes at 30°. The phosphorolytic and hydrolytic activities are expressed as microequivalents of P esterified and of sugar hydrolyzed during the initial 10 minute period per ml. of the mixture. Preparation A, 30 mg. of dry bacteria per ml.; Preparation B, enzyme preparation from 30 mg. of dry bacteria per ml partially purified by extraction and treatment with ammonium sulfate.

Substrate	Preparation A		Preparation B	
	P uptake	Sugar hydrolyzed	P uptake	Sugar hydrolyzed
Melibiose.	0.0	25.5	0.0	0.9
Sucrose . . .	13.6	8.2	3.5	0.1
Raffinose..	7.3	*	0.3	*

* Not determined.

For further proof that the phosphorylase produced by bacteria grown with raffinose is identical with that found in cells grown with sucrose, the enzyme preparation was treated with ammonium sulfate as previously described (3). From the last two columns in Table II it will be seen that while about a quarter of the phosphorylase activity towards sucrose survived the treatment, over 98 per cent of the invertase and over 96 per cent of the melibiase were removed or destroyed. The precipitated preparation caused very little phosphate uptake with raffinose, indicating that a preliminary hydrolysis of this sugar is necessary before phosphorolysis can occur.

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[1] Non-reducing; completely hydrolyzed in 7 minutes at 100° in N HCl to yield 1.0 (±0.001 equivalent of glucose (identified as glucosazone) per 1.0 equivalent of phosphate); specific rotation $[\alpha]_D = +81°$ (in water, $C = 1$); converted to starch by crude potato phosphorylase preparation.

Demonstration of Phosphorolysis in Vivo—Since no phosphorolysis of melibiose could be observed with dry cell preparations, it would seem that the decomposition of this sugar starts with its hydrolysis by an intracellular enzyme. Yet, under certain conditions, melibiose was shown to be oxidized more rapidly than its hexose constituents by intact cells. This might be due either to its more rapid penetration through the cell membrane or to a different mode of action of the bacteria on the disaccharide. It appeared possible that a phosphorolytic decomposition of melibiose could occur *in vivo* without being demonstrable *in vitro*, either because the necessary enzyme does not survive the dehydration of cell preparations or because a preliminary phosphorylation of the disaccharide must occur. In either case, the formation of glucose-1-phosphate or its galactose analogue could be expected as a product of phosphorolysis. For this reason, it seemed interesting to search for glucose-1-phosphate in intact cells, and to compare the relative amounts of this intermediate appearing during the oxidation of various substrates.

For this purpose, whole cells were inactivated while oxidizing various sugars, the phosphate esters were extracted, and the fraction representing glucose-1-phosphate was estimated. Although the presence of this ester *in vivo* was not established by absolute identification, it was very strongly indicated by the properties of the phosphate ester fraction tested. In addition, excellent agreement was obtained between the experiments with whole cells and those with dry cell preparations. The glucose-1-phosphate was determined as that fraction of the esterified phosphate which is soluble as a barium salt in water but insoluble in 67 per cent alcohol, which is not readily hydrolyzed by acid or alkali or decomposed with hypoiodite at room temperature, but which is hydrolyzed in 7 minutes at 100° with N HCl. These specifications would almost entirely eliminate adenylic acid, adenosine triphosphate, glucose-6-phosphate, fructose-6-phosphate, fructose diphosphate, triose phosphate, phosphopyruvic and phosphoglyceric acids, as well as acetyl phosphate. The bacteria were grown with raffinose as substrate, harvested by centrifugation, starved overnight by aeration in phosphate buffer, washed, and resuspended in M/900 buffer at pH 6.64 to make a 15 per cent suspension on the basis of dry weight. Aliquot portions of this suspension were then allowed to oxidize various substrates in an atmosphere of oxygen with constant agitation for 20 minutes at 30°. $\frac{1}{6}$ volume of 24 per cent trichloroacetic acid was added to the suspensions and the cells extracted for 2 hours at 4°. The cells were removed by centrifugation and reextracted with 4 per cent trichloroacetic acid, after which they were centrifuged out and the supernatants combined. Sufficient 2.5 N NaOH was added to the extract to give a faint pink color with phenolphthalein, $\frac{1}{10}$ volume of M barium acetate was then added, and enough alcohol to make the final concentration 67 per cent. After 24 hours at 4°, the precipitate was centrifuged down and dried *in vacuo* over P_2O_5 at room temperature. It was resuspended in a small volume ($\frac{1}{8}$ the volume of the original bacterial suspension) of 0.1 N acetic acid and neutralized with NaOH as before. $\frac{1}{20}$ volume of M barium acetate was added and precipitation of insoluble barium salts from aqueous solution allowed to proceed at 4° for 24 hours. The precipitate was removed by centrifugation and an excess of sodium sulfate added to the supernatant. After removal of the barium sulfate the supernatant was treated with 0.5 N NaOH for 15 minutes at room temperature, whereupon iodine was added to a final concentration of N/30. After 5 minutes, the excess iodine was reduced with sulfite and the solution neutralized and centrifuged. An aliquot was analyzed for inorganic phosphate without preliminary hydrolysis, while another aliquot was hydrolyzed for 7 minutes at 100° with N HCl. The difference was presumed to represent chiefly the glucose-1-phosphate fraction of the phosphoric esters.

TABLE III

Intracellular Accumulation of Glucose-1-phosphate in Vivo

Relative amounts of phosphoric esters in the fraction containing glucose-1-phosphate found in extracts of intact cells oxidizing various substrates (see the text).

Substrate	P fraction per gm bacteria (dry weight)
	microequivalents
None	0.6
Glucose	0.9
Melibiose	1.4
Sucrose	5.2
Raffinose	7.5

Although the recovery was not intended to be entirely quantitative, the exact similarity of treatment of all samples insured at least a basis for their comparison. In addition, preliminary experiments with pure glucose-1-phosphate showed better than 95 per cent recovery after similar treatment.

The results of an experiment in which the phosphoric esters were extracted from bacteria oxidizing different substrates are shown in Table III. It appears that only with raffinose and sucrose is there an appreciable accumulation of glucose-1-phosphate. No evidence for a phosphorolysis of melibiose *in vivo* could be found. The greater concentration of glucose-1-phosphate observed with raffinose than with sucrose did not seem to be fortuitous, since a repetition of the experiment yielded almost identical results.

DISCUSSION

From the experiments reported above it seems fair to conclude that the first step in the utilization of raffinose by *Pseudomonas saccharophila* involves chiefly its hydrolysis by intracellular melibiase. This is followed by the phosphorolytic and hydrolytic decompositions of the resultant sucrose. The reactions involved may be summarized by the following equations

$$\text{Raffinose} + H_2O \xrightarrow{\text{melibiase}} \text{sucrose} + \text{galactose} \tag{1}$$

$$\text{Sucrose} + \text{inorganic phosphate} \xrightarrow{\text{phosphorylase}} \text{glucose-1-phosphate} + \text{fructose} \tag{2}$$

$$\text{Sucrose} + H_2O \xrightarrow{\text{invertase}} \text{glucose} + \text{fructose} \tag{3}$$

In addition the raffinose may to a lesser extent be hydrolyzed by invertase, and the resultant melibiose further broken down by melibiase as follows:

$$\text{Raffinose} + H_2O \xrightarrow{\text{invertase}} \text{melibiose} + \text{fructose} \tag{4}$$

$$\text{Melibiose} + H_2O \xrightarrow{\text{melibiase}} \text{glucose} + \text{galactose} \tag{5}$$

It seems probable that the bulk of the metabolized raffinose is decomposed in accordance with equations (1) and (2). The melibiase and the combined phosphorylase and invertase activities of the dry cell preparations were found to be sufficient to account for from 5 to 8 times the observed rates of utilization of melibiose, sucrose, and raffinose by intact cells. Such comparison, however, is not entirely fair, since the substrate concentrations used in determining these activities were very much greater than the probable substrate concentrations inside the cells. No attempt was made to study either the internal concentrations or the kinetics of the enzymes.

The studies here reported still fail to explain why monosaccharides are attacked with greater difficulty than the complex sugars by bacteria accustomed to the latter. The lack of evidence for any but a hydrolytic cleavage of melibiose suggests that the rapid utilization of this sugar under certain conditions is not due to a special phosphorolytic mechanism. Experiments are now in progress which, it is hoped, may clarify the problem.

I wish to thank Dr. H. A. Barker and Dr. W. Z. Hassid of the University of California for their generous advice and assistance in the present work.

SUMMARY

1. Intact cells of *Pseudomonas saccharophila* grown with raffinose as substrate could oxidize raffinose and sucrose more rapidly than melibiose or any of the hexose constituents of raffinose separately or together, provided the compounds were made available in low concentration. When supplied in high concentration, melibiose was utilized very rapidly, while the monosaccharides were still oxidized relatively slowly.

2. Practically no hydrolytic or phosphorolytic enzymes catalyzing the breakdown of raffinose, melibiose, or sucrose were found in the medium after bacterial growth had taken place.

3. The presence of an active intracellular melibiase was demonstrated in a study of dry cell preparations. In addition, the cells contained invertase and a phosphorylase which was active towards sucrose but not towards raffinose. No direct phosphorolysis of either raffinose or melibiose could be shown.

4. The occurrence of phosphorolysis *in vivo* was indicated by the demonstration of a phosphoric ester having the properties of glucose-1-phosphate in respiring cells. Semiquantitative estimation of this compound substantiated the view that it originates mainly through the action of sucrose-phosphorylase. There was no evidence for a phosphorolytic breakdown of melibiose *in vivo*.

BIBLIOGRAPHY

1. Doudoroff, M., *Enzymologia*, **9**, 59 (1940).
2. Doudoroff, M., Kaplan, N., and Hassid, W. Z., *J. Biol. Chem.*, **148**, 67 (1943).
3. Doudoroff, M., *J. Biol. Chem.*, **151**, 351 (1943).
4. Hassid, W. Z., Doudoroff, M., and Barker, H. A., *J. Am. Chem. Soc.*, **66**, 1416 (1944).
5. Doudoroff, M., Hassid, W. Z., and Barker, H. A., *Science*, **100**, 315 (1944).

THE MICRODETERMINATION OF CERTAIN ALKALOIDS AND OTHER BASES BY PHOTOMETRIC TURBIDIMETRY

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Recent methods (5, 14, 17, 21) which have been proposed for the estimation of quinine in biological materials have in common the disadvantage of non-specificity, although the accuracy of estimation by each is adequate for rigidly controlled biological experiments if there is assurance that the absence of interfering substances obtains. The failure of these methods to reveal the presence of interfering substances in blank determinations on normal animal tissues or excreta does not confer unquestionable validity on the results which are obtained when the same methods are applied to tissues or excreta of an animal to which quinine has been administered unless there is established the complete lack of response of the methods to all metabolic products of the alkaloid. This criticism becomes more significant when it is recognized that much uncertainty exists with respect to the mechanism by which quinine performs its antimalarial action and that there is evidence to support both a direct action by the drug itself (19) and also an indirect action due to its metabolic decomposition products (23). Other uncertainties in the interpretation of data remain even if the action of quinine is direct, since its complete purification is tedious and expensive and the commonly employed grades of quinine are generally contaminated with other cinchona alkaloids. Moreover, the use of mixtures of cinchona alkaloids is supported by the present effort to make totaquine, a preparation of which the composition is loosely defined (20), an acceptable substitute for quinine of which the present source is limited.

This study was made to clarify the interpretation of measurements of quinine made by a method proposed by one of us (17), to define related substances which interfere (16), and to extend the application of the procedure to the assay of certain other substances.

EXPERIMENTAL

Chemicals[1]—The compounds which were studied are as follows: cinchona alkaloids (cinchonine, cinchonidine, hydroquinidine, quinine, quinidine, quitenine, and totaquine); other alkaloids (caffeine, cocaine, colchicine, morphine, narcotine, nicotine, strychnine, and theobromine); synthetic antimalarials (atabrine and plasmochin); compounds structurally common to part of the quinine molecule (pyridine and quinoline); vitamins (choline, nicotinic acid, pyridoxine, riboflavin, and thiamine); and miscellaneous nitrogenous substances (antipyrine, cinchophen, novocaine, sodium barbital, proteose-peptone, egg albumin, and serum albumin).

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* Part of the data in this report is abstracted from the thesis submitted by Dorothy Plonk Lewis to the Graduate Faculty of the University of North Carolina in partial fulfilment of the requirements for the degree of Master of Science, in June, 1943.

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[1] Appreciation is expressed to Merck and Company for samples of hydroquinidine, nicotinic acid, pyridoxine, quitenine, riboflavin, and thiamine and to the Winthrop Chemical Company for samples of atabrine and plasmochin.

A commercial grade of each of these was compared with purified quinine by the analytical method of Kyker, Webb, and Andrews (17). Most samples were of U. S. P. or equivalent quality and some were of reagent quality. Four samples of quinine, six of quinidine, and two each of both cinchonine and cinchonidine were used and each sample of a kind was obtained through a different source of supply which may represent a quality of product different from the others of its kind.

Preparations—A stock solution containing approximately 1000 mg. per liter was prepared for each sample by dissolving about 0.2000 gm. of the material which had been dried to constant weight at 105–110° in 200 ml. of 0.030 N hydrochloric acid solution. If any evidence of decomposition was observed, another sample was dried *in vacuo* over phosphoric anhydride. Choline, proteose-peptone, egg albumin, and serum albumin were dissolved to the approximately correct concentration and the resulting solutions were analyzed by the Kjeldahl method for total nitrogen from which the solute concentration was calculated.

Standard solutions in series were prepared by consecutive dilutions of each stock solution with an equal amount of 0.030 N hydrochloric acid until standards containing 500, 250, .. 1.0 mg. of solute per liter were obtained.

Observations—A concentration curve was obtained by plotting the galvanometer reading against the concentration of the solute in the series of standard solutions. The instrumentation consisted of an Evelyn photoelectric colorimeter and was operated with Filter 400. The procedure which was adopted is identical in every detail with that which is described under "Standard concentration curves" in the method under examination (17).

The extractability of the solute from alkaline aqueous samples was observed by subjecting portions from one of the standard solutions of convenient concentration to continuous ether extraction and determining the quantity extracted by applying the photelometric method for determining quinine with silicotungstic acid (17). The percentage of removal by the extraction was evaluated by the previously determined concentration curve for the same compound.

The specific optical rotation was determined for each of the optically active substances which are listed above by observing the rotation of each of the standard solutions which was of adequate concentration to give an observed rotation of significant magnitude. All determinations were made with a Schmidt and Haensch half shadow polarimeter reading to ±0.01°, with monochromatic D light from an electric sodium vapor lamp and 2 dm. tubes.

The composition of the silicotungstates was determined in each case by the general procedure which is described below. A 50 ml. portion of each stock solution was treated with a 2:1 molar excess of 10.0 per cent silicotungstic acid. The resulting suspension was digested for several hours on a steam plate, stored in an ice box overnight, filtered through a weighed No. 4 Jena glass crucible, washed several times with small portions of 0.03 N hydrochloric acid, and dried to constant weight at 105°. A portion of the dry silicotungstate was transferred to a weighed porcelain crucible and ignited in an electric furnace at 700° for consecutive 30 minute periods until constant weight was reached. The weight of the sample of silicotungstate for ignition was checked by difference from both the Jena crucible and the porcelain crucible, since the former was weighed after drying at 105° and the latter after igniting at 700°. The temperature of ignition was selected as the optimal temperature which minimizes incomplete dehydration of the silicon oxide on the one hand and volatilization of tungsten oxide on the other (22). Calculations were made according to

$$\frac{\text{Mol. wt }(SiO_2\cdot 12WO_3)\times \%\text{ base}}{\%\ (SiO_2\cdot 12WO_3)\times \text{mol wt. of base}} = \frac{\text{moles base}}{\text{mole }(SiO_2\cdot 12WO_3)}$$

If the solution of this equation failed to give a small whole number of moles of base per mole of acid oxides, the positive fractional remainder was expressed as water of composition or crystallization which was stable to 105° drying.

DISCUSSION

Atabrine, cinchophen, colchicine, plasmochin, riboflavin, and theobromine are omitted from further discussion because either the interference of the yellow color of their solutions (with Filter 400) or the difficulty in preparing a stock solution due to limited solubility eliminated these from this study. Also, nicotinic acid, pyridoxine, and sodium barbital are not considered beyond this point because no insoluble silicotungstate resulted from either of these, even when the concentrated stock solution was treated with the reagent. In the group of cinchonas which were studied quinidine differed from the others in that it gave a silicotungstate precipitate which appeared as a very finely divided irridescent crystalline solid which settled rather rapidly and did not offer sufficient stability for taking turbidimetric readings.

The curves which are presented in Figs. 1, 2, and 3 pertain to those substances which simulate quinine in their response to the analytical method for quinine. The accuracy of this analytical method, when applied to any one of these compounds, depends on the slope of the curve and the stability of the turbidity. The latter is the measured factor that indicates the concentration of the substance in question and it is reflected by the regularity with which the data approach the curve. As in the case of quinine (17), the curves do not represent a true expression of the Lambert-Beer law, although there is an approximation to this expression for certain segments of some of the curves. Consequently for each compound the preliminary construction of a concentration curve, which may be used for the estimation of unknown quantities of the same compound, is prerequisite to the quantitative use of this method for the analysis of any of the compounds to which it can be applied.

Hydroquinidine, quinine, cinchonidine, cinchonine, quitenine, and totaquine present in each case results which are very similar to the others. This is not unexpected, since the cinchonas present such close structural similarities. Since different samples of a cinchona, obtained through separate sources and of varying purity, will have as the most probable impurity some other cinchona, the analytical results for all such samples will be similar regardless of their purity. Each of the above cinchonas, as well as nicotine and thiamine which are also shown in Fig. 1, can be measured as accurately as quinine (17) provided that each is not contaminated by other interfering substances and is in solution in 0.030 N hydrochloric acid. A search for optimal conditions individual to each should improve the method for that particular compound over that which has been attained for it by imposing the optimal conditions for quinine. The amount of nicotine which is present in human urine of a moderate or heavy smoker (7) does not influence the results for quinine during a metabolic period because the urinary concentration of quinine after a dose and the sensitivity of the method for quinine are sufficient to require so large a dilution factor before determination that the level of excretion of nicotine is not detectable by the method at the final dilution. Trace amounts of nicotine can be estimated by taking up the extracted or distilled alkaloid in a volume of 0.030 N hydrochloric acid which is less than that of the sample. The rapidity and economy of this micromethod for nicotine over the gravimetric procedure which also employs silicotungstic acid reagent (22) should solicit its use whenever a micro sample and the tolerable error may warrant. The microestimation of thiamine in a vitamin preparation which contains no interfering compounds may be accomplished within an error of approximately ±3 per cent by the brief operations of dissolving in acid, preparing the turbidity, and taking a reading. There would be no interference by nicotinic acid, pyridoxine, and choline if these members of the vitamin B complex were present with thiamine, but riboflavin would interfere on a colorimetric basis and it would require a preliminary separation or destruction before the estimation of thiamine could be accomplished.

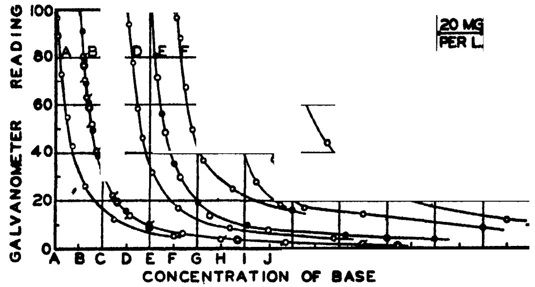

FIG. 1. The photelometric response of the silicotungstate turbidities of the cinchonas, nicotine, and thiamine as a function of the concentration of each. Abscissa values are plotted on the same scale, but zero concentration on this axis begins at a different point for each compound as follows: hydroquinidine (*A*), quinine (*B*), cinchonidine (*D*), cinchonine (*E*), quitenine (*F*), totaquine (*H*), nicotine (*I*), and thiamine (*J*). The curves for these compounds are lettered with the same symbol as that which designates the point of origin on the abscissa.

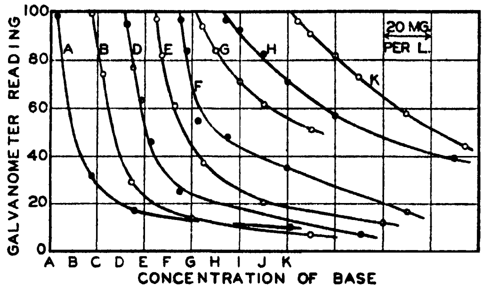

FIG. 2. The photelometric response of the silicotungstate turbidities of quinoline, novocaine, certain alkaloids, and certain proteins as a function of concentration. Abscissa values are plotted on the same scale but zero concentration on this axis begins at a different point for each compound as follows: novocaine (*B*), strychnine (*D*), narcotine (*E*), cocaine (*F*), serum albumin (*G*), egg albumin (*H*), proteose-peptone (*K*); *A* designates 30 mg. per liter of quinoline. The curves for these compounds are lettered with the same symbol as that which is assigned to its origin on the abscissa.

The compounds which were found to respond like quinine, but only at slightly higher concentrations, are presented in Fig. 2. The response which was recorded for both primary and secondary proteins is of primary significance in the application of this method to the analysis of quinine, or of any other compound which reacts similarly, when the compound is present in a biological sample or protein-bearing mixture. An extraction which includes no trace of protein in the separated fraction is strictly imposed. Strychnine, narcotine, and novocaine give perceptible turbidities at 2, 4, and 8 mg. per liter respectively. Interference by novocaine has been recorded (24) in the analysis of the urine of dogs when a dose of quinine and the collection of the specimen had been preceded by novocaine anesthesia and surgical treatment. The curve which is presented for novocaine quantifies its interference. The data which describe cocaine do not agree closely with a regular curve and are similar to data from other cases in which unstable turbidities resulted from treatment with silicotungstic acid reagent. Quinoline gives no turbidity under 30 mg. per liter. It is not likely to occur as an impurity in any of the substances which are shown to be adaptable to this procedure and it was studied only because of its structural relationship to part of the cinchona nucleus.

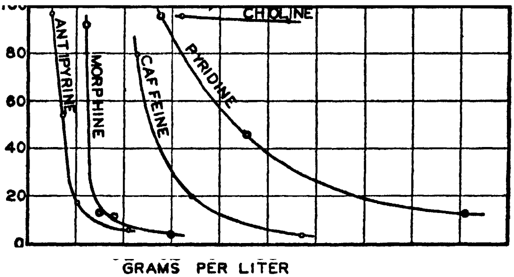

FIG. 3 The photelometric response of the silicotungstate turbidities of antipyrine, morphine, caffeine, pyridine, and choline as a function of concentration.

At relatively high concentrations, antipyrine, morphine, caffeine, pyridine, and choline produce turbidities with silicotungstic acid and the minimum concentration which yields a turbidity increases for these in the order of their listing (Fig. 3). Turbidities resulting from choline are hardly detectable below 0.5 per cent under these conditions. The minimum concentration for each of these is sufficiently high to permit disregarding them when dealing with those compounds which are described in Figs. 1 and 2.

One of two compounds may be determined in the presence of the other when both possess qualitatively and quantitatively similar concentration curves if the other is not extractable under the conditions of the continuous ether extraction. An evaluation of this property showed micro amounts of the cinchonas, with the exception of quitenine, to be extracted to the extent of 95 to 100 per cent. Quinidine is extracted as well as the others, presumably, but the instability of its silicotungstate turbidity renders impossible a rigid definition of its concentration curve and therefore its extractability could not be determined accurately. Quitenine is not extracted to any detectable degree. Its dissociation curve (2) indicates that it is present mainly in the anionic form at the pH (excess alkali to phenolphthalein) at which the extraction was carried out and little or no extraction of the salt form with ether was anticipated. Other substances which were not removed from the aqueous phase by the ether extraction are thiamine, cocaine, and the proteins, both primary and secondary. Approximately two-thirds of narcotine and 80 to 90 per cent of both novocaine and nicotine were removed during the standardized period under the conditions by which the continuous extraction was performed.

Specific rotations which have been reported (1, 3, 6, 11) for the cinchonas and certain other alkaloids which are included in this study were not measured under the same conditions from which the data presented in Table I were obtained. In this study the solvent was 0.030 N hydrochloric acid throughout and the maximum concentration was approximately 0.1 per cent. The values in Table I remained constant for part of the alkaloids when the concentration was decreased considerably and in the case of others consistent variations with concentration were observed. On the more dilute standards the specific rotations were calculated from very small observed rotations but the small magnitude of error by the instrument which was used makes these values significant. For those compounds which exhibited consistent variations, the influence of concentration on specific rotation is shown in Fig. 4.

The properties of silicotungstic acid which allow it to serve as a general precipitant for alkaloids and other basic substances have been recognized by many investigators. The acid acts usually as a tetrabasic reagent for these substances. No silicotungstate of a weak organic base has ever been found to contain more than 4 moles of the base per mole of the acid and it is extremely rare to find a silicotungstate in whose formation the parent acid has not exercised fully its tetrabasicity. Controversial reports have been made concerning the influence of the composition and conditions of the system, from which the silicotungstate is precipitated, on the composition of the salt. In some instances greater concentrations of electrolytes in the system have influenced the precipitation of silicotungstates whose compositions exhibit a smaller ratio of base to acid than those which are formed from the same reactants in systems of lower electrolyte concentration. These previous reports (10, 15, 18) prompted the analysis of the silicotungstates which were prepared in this study, since each was precipitated from a solution of its base in 0.030 N hydrochloric acid. The data are recorded in

TABLE I

Specific Rotation of Certain Alkaloids in 0.030 N Hydrochloric Acid

Compound	Specific rotation	Concentration	Source and description
		gm. per 100 ml.	
Cinchonidine base	−185	0.100	Eimer and Amend (pure, E11)
" sulfate	−170	0.088	Kahlbaum
Cinchonine hydrochloride .	+239	0.067	Eimer and Amend (pure, E11)
" sulfate	+256	0.079	" " " (N. F. VI, E11)
Cocaine hydrochloride .. .	−80	0.100	Merck
Hydroquinidine base	+295	0.100	" (for investigation only)
Morphine sulfate	−100	0.060	"
Narcotine base	+45	0.100	Kahlbaum
Quinidine " ..	+326	0.127	Mallinckrodt (crystals)
" "	+321	0.112	Eimer and Amend (crystals)
" "	+320	0.109	Kahlbaum (four recrystallizations)
" hydrochloride	+330	0.098	Eimer and Amend (pure, E11)
" sulfate	+316	0.065	" " " (U. S. P.)
" "	+330	0.148	Merck (U. S. P.)
Quinine base	−284	0.110	From Merck recrystallized sulfate
" hydrochloride .	−285	0.140	Mallinckrodt (U. S. P.)
" sulfate	−292	0.107	Merck (U. S. P.)
" "	−290	0.097	Eimer and Amend (U. S. P.)
Quitenine ..	−312	0.088	Merck
Strychnine	−44	0.101	" (N. F., crystals)

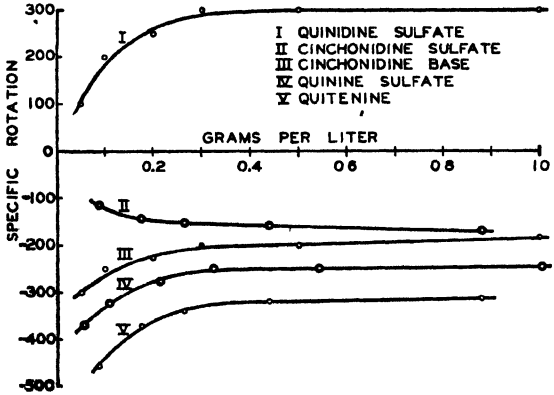

FIG. 4. The specific rotation of certain cinchona alkaloids in 0.030 N hydrochloric acid as a function of concentration.

Table II and the composition is expressed as a molar ratio of base to acid to water. With the exception of small differences for the water of composition, the molar ratios which were determined agree with earlier studies for the following: antipyrine (12), caffeine, cinchonine, and colchicine (10), cocaine, morphine, quinine, and strychnine (10, 15), narcotine (8, 10), nicotine (4, 10, 22), and pyridine and quinoline (13). 4 moles of base have been reported to combine with 1 mole of acid in the preparation of the silicotungstate of atabrine (9) and the silicotungstate of choline (18), whereas the results of this study indicate 2 and 3 moles of base, respectively, per mole of acid. No previous reports have been made with which to compare the composition of the silicotungstates of cinchonidine, hydroquinidine, novocaine, quinidine, quitenine, and thiamine which are also included in Table II. The characteristics of silicotungstic acid as an alkaloidal reagent which have been described heretofore are borne out by this study.

TABLE II

Analysis and Composition of Silicotungstates of Certain Alkaloids and Other Bases

Compound	Sample	Residue	SiO_2 $12WO_3$	Molar ratio Base to acid to water
	gm.	*gm.*	*per cent*	
Antipyrine	0.1970	0.1528	77.25	4:1:4
Atabrine	0.1741	0.1367	78.52	2:1:0
Caffeine	0.2675	0.2155	80.56	3:1:6
Choline	0.1424	0.1231	86.45	3:1:5
Cinchonidine	0.2747	0.2233	81.29	2:1:4
Cinchonine	0.8831	0.7170	81.20	2:1:4
Cocaine	0.0184	0.0123	66.85	4:1:11
Colchicine	0.0374	0.0241	64.44	4:1:0
Hydroquinidine	0.2647	0.2111	79.75	2:1:4
Morphine	0.0579	0.0417	72.02	4:1:0
Narcotine	0.0947	0.0611	62.73	4:1:2
Nicotine	0.4662	0.4130	88.59	2:1:3
Novocaine	0.1405	0.1184	84.25	2:1:0
Pyridine	0.3500	0.3114	88.97	4:1:2
Quinidine	0.2600	0.2074	79.80	2:1:4
Quinine	0.2378	0.1908	80.23	2:1:3
Quinoline	0.4154	0.3520	84.84	4:1:0
Quitenine	0.2476	0.1956	79.00	2:1:4
Strychnine	0.1177	0.0807	68.56	4:1:0
Thiamine	0.1203	0.0984	81.79	2:1:6

SUMMARY

The study of a previously proposed method for the microestimation of quinine has been extended to seven cinchona products, eight other alkaloids, two synthetic antimalarials, five members of the vitamin B complex, three proteins, and six other nitrogenous products. With the exception of quinidine all of the cinchonas, nicotine, and thiamine respond qualitatively and quantitatively in a similar manner. At slightly higher concentrations cocaine, narcotine, novocaine, strychnine, egg albumin, serum albumin, proteose-peptone, and quinoline give similar quantitative responses. Quitenine, thiamine, cocaine, and the proteins are not extractable. Narcotine, nicotine, and novocaine show a relatively high but an incomplete extraction. Specific rotations were determined for the optically active compounds in 0.030 N hydrochloric acid solutions. The composition of the silicotungstate which was prepared from each of twenty bases was determined. The method which was originally proposed for quinine has been shown to be applicable to different organic bases.

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BIBLIOGRAPHY

1. Andrews, J. C., *Ind. and Eng. Chem., Anal. Ed.*, **14**, 543 (1942)
2. Andrews, J. C, and Cornatzer, W E, *J. Elisha Mitchell Sc Soc*, **59**, 177 (1943).
3. Andrews, J. C., and Webb, B. D., *Ind. and Eng. Chem, Anal Ed.*, **13**, 232 (1941).
4. Bertrand, G., and Javillier, M., *Ann. chim. anal. et. chim. appl*, **14**, 165 (1909).
5. Brodie, B. B., and Udenfriend, S., *J. Pharmacol. and Exp. Therap*, **78**, 154 (1943).
6. Carr, F. H., and Reynolds, W. C., *J. Chem Soc.*, **97**, 1328 (1910).
7. Corcoran, A. C., Helmer, O M., and Page, I. H., *J. Biol Chem*, **129**, 89 (1939).
8. Duquénois, P., and Ellert, M., *Bull. Soc. chim.*, **6**, 1582 (1939)
9. Gorgainova, N. S., *Farm. i Farmakol*, No. 10, 15 (1937).
10. Heiduschka, A., and Wolf, L., *Schweiz. Apoth Ztg*, **58**, 213, 229 (1920)
11. Henry, T. A, The plant alkaloids, Philadelphia, 3rd edition (1939)
12. Javillier, M., *Bull. sc. pharmacol*, **19**, 70 (1912).
13. Kahane, E., and Kahane, M, *Bull. Soc. chim*, **3**, 621 (1936)
14. Kelsey, F E, and Geiling, E M. K, *J. Pharmacol. and Exp Therap.*, **75**, 183 (1942).
15. Klyachkina, B. A, Strugatzkii, M K, and Zilberg, F. D, *Byull. Nauch -Issledovatel Khim.-Farm. Inst.*, 203 (1931)
16. Kyker, G. C., *Federation Proc*, **1**, pt. 2, 120 (1942)
17. Kyker, G C, Webb, B. D, and Andrews, J. C, *J. Biol. Chem*, **139**, 551 (1941).
18. Lematte, L., Boinot, G., Kahane, E, and Kahane, M, *Compt rend. Acad.*, **191**, 1130 (1930).
19. Marshall, E. K., *Physiol. Rev.*, **22**, 190 (1942).
20. Meleney, H. E., *J. Nat. Malaria Soc.*, **2**, 77 (1943).
21. Prudhomme, R. O., *J. pharm. et chim.*, **1**, 8 (1940).
22. Spies, J. R., *Ind. and Eng. Chem., Anal. Ed.*, **9**, 46 (1937)
23. Waletzsky, E., and Brown, H. W., *J. Nat. Malaria Soc*, **2**, 53 (1943)
24. Webb, B. D, Dissertation, University of North Carolina (1941).

THE DIFFUSION OF ORGANIC IONS AND THE EINSTEIN-SUTHERLAND RELATIONSHIP

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The Einstein-Sutherland equation, $D = (RT)/(6\pi\eta_0 rN)$, ((1, 2) (3) p. 280) relating the diffusion constant, D, to the molecular radius of the diffusing particle, r, and the viscosity of the medium, η_0, has frequently been employed to estimate the molecular weight of large spherical particles in dilute solution. When applied to particles of the size of protein molecules, this relationship has been found to provide a close approximation of the molecular weight as determined by other methods (4). As the Einstein-Sutherland equation requires that the solute molecules be large compared to the solvent molecules, it is commonly agreed that this equation does not apply to substances of small molecular weight. Thus from certain theoretical considerations Euler (5) postulated that the diffusion coefficient for small molecules is inversely proportional to the square root of the molecular weight instead of the cube root as given by the Einstein-Sutherland equation. Similarly, Sutherland (2) has calculated that the numerical factor approaches 4 rather than 6 when the solute spheres are of the same order of size as the solvent spheres.

In the present paper we have measured the diffusion coefficients of 2 relatively small organic molecules of known structure, disodium β-glycerophosphate and disodium guanylate, to find how closely the Einstein-Sutherland equation may be expected to hold for particles of this size. To eliminate the electrical effects due to the diffusion of charged particles, the diffusion rate of each compound was determined in the presence of varying concentrations of supporting electrolyte.[1] As shown by Dean (6), the diffusion coefficient of an ion approaches the value it would have in the absence of electrical effects produced by ions of opposite charge, when the ratio, R, of the conductivities of the supporting electrolyte to the supporting electrolyte plus diffusing ions approaches unity. From the graph of diffusion coefficient (corrected for viscosity) as a function of the conductivity ratios, the theoretical values for the uncharged molecules were obtained by extrapolation. The calculation of molecular weights from these values showed, within rather wide limits of error, that β-glycerophosphate and guanylate ions obey the Einstein-Sutherland diffusion law when sufficient supporting electrolyte is present to reduce the electrical effects to a negligible value.

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[1] A supporting electrolyte is defined as one (either a single salt or a mixture) that is initially present at the same concentration throughout the diffusion system.

EXPERIMENTAL

Diffusion was measured at 25° through sintered glass disks in double ended diffusion cells described by McBain and Dawson (7). Calculations of the diffusion coefficient were made by means of a nomograph based on the equation for diffusion in these cells derived by Vinograd and McBain (8). All solutions were made up with at least 70 per cent boiled distilled water to avoid accidental formation of bubbles in the membranes. Five cells in all were used, and each system was measured in at least two different cells. The cell constants were frequently redetermined with 0.1 N KCl. We assume the value for the diffusion coefficient of 0.1 N KCl to be $D = 1.631$ sq. cm. per day at 25° obtained from the value found by Cohen and Bruins (9) at 20°.

The concentration of diffusing ion used both for the disodium β-glycerophosphate and the disodium guanylate was 0.01 M. The amount diffused over a given period as well as the original concentration was determined by analyses for total phosphate. In most of the experiments with glycerophosphate the colorimetric method of King (10) was used. The colored solutions were compared in a Klett-Summerson photoelectric colorimeter. Because more precise analytical results were obtained by the gravimetric method of Embden (11), this was adopted in the later experiments with disodium guanylate.

The supporting electrolyte consisted of borate buffer prepared by the dilution of a stock solution at pH 7.6 which was 1 M with respect to borate and 0.45 with respect to chloride ((12) p. 209). The respective dilutions used were 0.05, 0.1, and 0.2 M with respect to borate.[2] The glycerophosphate solutions for diffusion were prepared by the dilution of a 0.1 M disodium β-glycerophosphate solution and 1 M borate buffer to give the concentrations mentioned.

The guanylate solutions were prepared in two ways. In the first method, crystalline trisodium guanylate obtained by the method of Steudel and Peiser (13) was neutralized with sufficient hydrochloric acid to give the disodium salt, the solution of which was then diluted with 1 M buffer and water to give solutions 0.01 M with respect to guanylate and 0.05, 0.1, and 0.2 M with respect to borate for the different solutions. In the second method, a preparation of lyophilized guanylic acid was used which had been obtained from the crystalline trisodium salt after precipitation with lead acetate and decomposition of the lead salt with hydrogen sulfide. A weighed quantity of the dried guanylic acid was neutralized with the theoretical amount of sodium hydroxide necessary to give the disodium salt, and the resulting solution was diluted with 1 M borate and water to give solutions that were 0.01 M with respect to guanylate and were 0.05, 0.1, and 0.2 M respectively with regard to borate. Sufficient sodium chloride was added to both the buffer solutions alone and the guanylate solutions prepared by the second method to make the respective solutions comparable in chloride content to those prepared by the first method.

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[2] Borate buffer was used because it has been found to prevent bacterial contamination in solutions which would otherwise support bacterial growth when allowed to stand for appreciable lengths of time at room temperature.

The pH of the diluted buffer solutions varied from 7.6 to 8.0, but in no case was the difference between buffer alone and buffer plus diffusing ion greater than 0.1 pH. At a pH value of 7.6 approximately 95 per cent of the β-glycerophosphate is present as divalent ion as determined by a titration curve for the 0.01 M solution. Similarly as given by Levene and Simms (14), guanylate is present almost entirely as divalent ion at pH 7.6.

Conductivities were obtained after the diffusion work was completed and were made on solutions duplicating as far as possible those used in the original work. Viscosity of the stronger buffer solutions was measured and the viscosity of the more dilute buffer solutions obtained by calculation, assuming that the change in relative fluidity is proportional to the concentration of the added salts. The viscosity corrections in no case exceeded 3 per cent.

Results

The diffusion coefficients of the β-glycerophosphate and of the guanylate ions in the various concentrations of supporting electrolyte are plotted against the conductivity ratio, *R*, in Fig. 1. Each value for the diffusion coefficient represents the average of from two to six determinations. The maximum standard deviation for the runs in which β-glycerophosphate was used was 0.46 sq. cm per second;[3] the corresponding value for the guanylate was 0.28 sq. cm. per second. Extrapolation of the data for a conductivity ratio of 1 gave values for the diffusion coefficient of the ions in the absence of electrical effects of 6.4 sq. cm. per second for the β-glycerophosphate and of 4.4 sq. cm. per second for the guanylate. The solid curve is the theoretical relationship between diffusion coefficient and the conductivity ratios calculated from these values and from the diffusion coefficients 10.0 and 8.4 sq. cm. per second obtained for the β-glycerophosphate and guanylate respectively in the absence of supporting electrolyte (6). As the values for the theoretical diffusion coefficients of unhydrated β-glycerophosphate and guanylate ions are 7.0 and 5.6 sq. cm. per second respectively, it is evident that the values obtained when the conductivity ratio, *R*, is 1 are between 10 and 30 per cent lower than those for the unhydrated molecules. Calculations of molecular volume from the diffusion coefficients, 6.4 and 4.4 sq. cm.^{2} per second for the β-glycerophosphate and guanylate respectively, gave values of 142 and 430 cc. As these molecular volumes correspond to β-glycerophosphate and guanylate ions containing 2 and 12 molecules of water of hydration respectively, the data suggest that these ions may be hydrated and that this factor accounts, at least in part, for the lower diffusion coefficients. As models of the diffusing ions showed that molecules of both compounds are nearly spherical, it is likely that the asymmetry effects can be neglected in comparison with hydration. While the experiments are not sufficiently precise to warrant the interpretation that the above values for the extent of hydration are exact, the results definitely support the Einstein-Sutherland equation for organic ions in the presence of sufficient supporting electrolyte to eliminate the electrical effects of the oppositely charged diffusing ions.

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[3] Values for the diffusion coefficients should be multiplied by 10^{-6}.

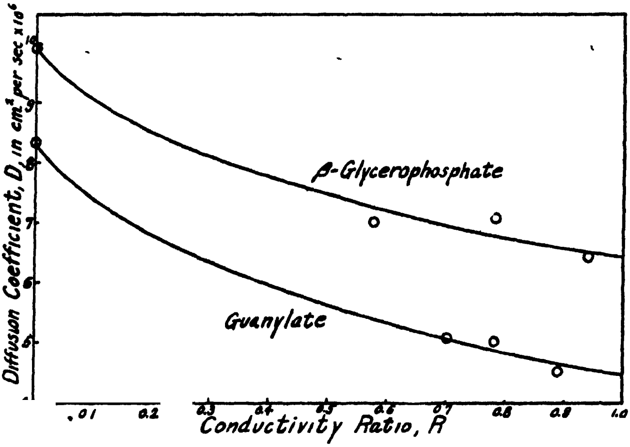

FIG. 1. The points are the diffusion coefficients of 0.01 M divalent β-glycerophosphate and 0.01 M divalent guanylate ions plotted as a function of the conductivity ratio, R, with borate buffer as the supporting electrolyte. The solid curves show the theoretical relationship as given by Dean (6).

The authors are indebted to John G. Pierce and Waldo Hanns for the analytical determinations.

SUMMARY

1. The diffusion of 2 organic divalent ions was measured in the presence of supporting electrolyte and the limiting value of the diffusion coefficient of the ions in the absence of electrical effects calculated.

2. The experiments support the use of the Einstein-Sutherland equation for organic ions with a molecular weight as small as 140.

BIBLIOGRAPHY

1. Einstein, A., *Z Elektrochem.*, **14,** 235 (1908).
2. Sutherland, W., *Phil. Mag.*, **9,** 781 (1905).
3. Bull, H. B., Physical biochemistry, New York, 280 (1943).
4. Williams, J. W., and Cady, L. C., *Chem. Rev.*, **14,** 202 (1934)
5. Euler, H., *Ann. Phys. u. Chem.*, **63,** 273 (1897).
6. Dean, R. B., *J. Am Chem. Soc*, in press.
7. McBain, J. W, and Dawson, C R., *Proc. Roy. Soc., London, Series A*, **148,** 32 (1935).
8. Vinograd, J. R, and McBain, J. W., *J. Am. Chem. Soc.*, **63,** 2008 (1941).
9. Cohen, E., and Bruins, H. R., *Z phys. Chem.*, **103,** 349 (1923); **113,** 157 (1924).
10. King, E. J., *Biochem. J.*, **26,** 292 (1932).
11. Embden, G., *Z. physiol. Chem.*, **113,** 138 (1921).
12. Clark, W. M., The determination of hydrogen ions, Baltimore, 3rd edition, 209 (1928).
13. Steudel, H, and Peiser, E., *Z. physiol. Chem.*, **114,** 201 (1921); **120,** 292 (1922).
14. Levene, P. A., and Simms, H S, *J. Biol. Chem*, **65,** 519 (1925).

ELECTROPHORETIC AND SALT FRACTIONATION OF THE SERUM PROTEINS OF NORMAL AND HYPOTHYROID RATS*

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(Received for publication, November 17, 1944)

In a previous communication (1) we reported that the sera of intact, normal rats of the Long-Evans strain, when examined electrophoretically in saline-sodium phosphate buffer at pH 7.4, contained little or none of the protein component usually designated as α-globulin. In contrast, sera taken from rats 21 days after hypophysectomy uniformly showed the presence of this component.

Earlier experiments (2) depending on salt fractionation methods had shown that the concentration of total serum globulin increased after thyroidectomy as well as after hypophysectomy and that such globulin elevation might be prevented by thyroid replacement therapy. It was therefore of interest to ascertain whether the sera of thyroidectomized rats showed the presence of α-globulin and whether this component accounted for at least a portion of the increase in total serum globulin. Likewise, it seemed probable that the sera of rats made hypothyroid (3) by thiouracil administration would show the presence of α-globulin.

The data reported in this paper, obtained from the sera of eight thyroidectomized, eleven thiouracil-fed, and eight additional untreated control rats, show that α-globulin does indeed appear in the serum of hypothyroid rats.

EXPERIMENTAL

A group of male rats of the Long-Evans strain was thyroidectomized when 3 to 4 months old. They were allowed to eat the stock diet (4) *ad libitum* and water was available in the cages at all times. During the postoperative period of 21 days the animals usually showed a small body weight loss, although some of them gained slightly. After this interval, the animals were anesthetized by intraperitoneal administration of sodium amytal, 10 mg. per 100 gm., and were bled by heart puncture. The blood was allowed to clot and the clear serum collected.

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* Aided by a grant from the Rockefeller Foundation, administered by Dr. P. E. Smith.

Another group of male rats of similar age and weight was rendered hypothyroid by *ad libitum* feeding of the stock diet containing 0.2 per cent 2-thiouracil.[1] After a 21 day period on this diet, the animals were bled as described above.

Electrophoretic analyses were made on aliquots of the serum after dilution with 2 volumes[2] of 0.02 M sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl and dialysis of the diluted serum against large volumes of the same buffer in the usual manner. The analyses were made in a Tiselius apparatus having a tall, single sectioned cell of 2 ml. capacity (5).

Serum proteins were also determined by the sodium sulfite fractionation method of Campbell and Hanna (6).

RESULTS AND DISCUSSION

A summary of the data is presented in Table I. As may be seen, these data, electrophoretic as well as salt fractionation, confirm earlier findings (2) that thyroidectomy leads to an increase in serum globulin concentration with little change in albumin concentration. The electrophoretic analyses further show that in every thyroidectomized rat the α-globulin concentration is elevated sufficiently to permit definite detection of this component under the conditions here used. In contrast, only four of the twenty (eight in this and twelve in the previous series (1)) normal rat sera analyzed electrophoretically have shown the presence of this protein component.

Typical electrophoretic patterns obtained with sera of normal and thyroidectomized rats are reproduced in Fig. 1. In some of the patterns for normal rat serum, as illustrated in Fig. 1, *A*, the curve did not return completely to the base-line between the albumin and β-globulin components, indicating the presence of electrophoretically heterodisperse protein in that region. In measuring the areas under the curves for purposes of estimating protein concentration, this protein was included partially with the albumin and partially with the β-globulin fractions. The γ-globulin component is appreciably higher in the serum from the thyroidectomized rats than that from the normals.

It may also be pointed out that the sera from some of the thyroidectomized rats contained an additional component which appeared in the patterns as a shoulder on the albumin curve (Fig. 1, *B*). On prolonged electrophoresis this separated into a distinct component. A similar additional component was also seen in the patterns of two of the four "normal" sera which showed the presence of α-globulin. Whenever this component appeared, it was included with the albumin in calculations of the relative concentrations of the various fractions.

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[1] The 2-thiouracil (deracil) was kindly supplied by Dr. S. M. Hardy of the Lederle Laboratories, Inc., Pearl River, New York.

[2] In the previous paper (1) of this series, it was erroneously stated that the sera were diluted with 3 volumes of buffer solution prior to dialysis. The procedure actually was identical with that indicated in the present paper; *i.e.*, dilution with 2 volumes of buffer followed by dialysis against a large volume of the same buffer.

TABLE I

Summary of Data on Sera from Normal, Thyroidectomized, Thiouracil-fed, and Hypophysectomized Rats

No. of rats	Salt fractionation, gm per cent				Electrophoretic fractionation*						
	Total protein	Albumin	Globulin	$\frac{\text{Albumin}}{\text{Globulin}}$	Total protein	Albumin	Globulins				$\frac{\text{Albumin}}{\text{Globulin}}$
							α	β	γ	Total	
20, normal	6.04 ±0.25†	3.86 ±0.23	2.18 ±0.19	1.79 ±0.22	581 ±42	415 ±30	‡	93 ±25	63 ±18	166 ±35	2.6 ±0.6
8, thyroidectomized	6.70 ±0.65	3.50 ±0.32	3.19 ±0.53	1.12 ±0.24	612 ±66	418 ±63	39 ±9	68 ±10	88 ±21	194 ±28	2.2 ±0.4
11, thiouracil-fed	7.03 ±0.49	4.40 ±0.25	2.64 ±0.26	1.68 ±0.15	636 ±60	455 ±36	44 ±9	76 ±16	61 ±13	181 ±24	2.6 ±0.4
6, hypophysectomized	5.76 ±0.23	3.20 ±0.21	2.56 ±0.22	1.25 ±0.17	535 ±106	337 ±88	29 ±16	72 ±20	97 ±19	198 ±36	1.7 ±0.5

* Expressed in arbitrary units derived from the descending patterns.

† Standard deviation, $\sqrt{\Sigma\,(x-\bar{x})^2/(N-1)}$.

‡ Only four of the twenty specimens showed the presence of α-globulin, the average of these four values being 26 ± 8.

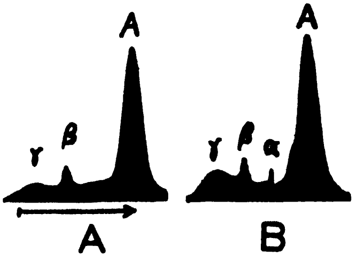

FIG. 1. Electrophoresis patterns of serum from (*A*) normal and (*B*) thyroidectomized rats. Buffer, 0.02 M sodium phosphate + 0.15 M NaCl at pH 7.4. Serum diluted with 2 parts of buffer

Thiouracil feeding, as was expected, also produces an increase in serum globulin level. The figures derived by both the salt fractionation and electrophoretic methods agree in showing that the increase in globulin level during thiouracil feeding is not as great as that resulting from thyroidectomy. Likewise both methods agree in showing that during the thiouracil feeding there is a significant increase in albumin concentration, whereas after thyroidectomy the albumin concentration remains stationary or decreases slightly. The reason for this difference in behavior of thyroidectomized and thiouracil-treated rats is unknown to us at the present time.

The fact that in the thiouracil-treated rats the concentrations of both the albumin and globulin components are above normal might be interpreted as being a result of hemoconcentration. Although such a concept agrees with the reported relation of blood volume to thyroid activity in man (for review of literature see (7)), the few hematocrit readings we have made do not support this possibility. In every case, the hematocrit values were found to be normal or below normal, indicating a normal or perhaps slightly increased plasma volume. If the plasma volume is indeed actually decreased, one must interpret our hematocrit values as indicating a coincident and proportionally greater decrease in total cell volume.

Similarly, the increased serum globulin concentration found after thyroidectomy may be interpreted as being due to hemoconcentration. Such a conclusion, however, is even more difficult than in the case of thiouracil-fed rats, for after thyroidectomy not only does the hematocrit reading fall, but the albumin concentration is consistently lower than in normal rats (2). Therefore, if the postthyroidectomy increase in serum globulin is due to a decrease in plasma volume, it must be assumed that there is a coincident, and proportionally greater, decrease in total red cell volume as well as in total circulating albumin. In this respect it is interesting to analyze the blood volume data of Gibson and Harris (7), who studied seventeen cases of severe human hyperthyroidism which showed substantial reduction of basal metabolic rate following treatment. In five of these seventeen cases, the decrease in basal metabolic rate was accompanied by an *increase* (1.8 to 11.5 per cent, average 4.8 per cent) in plasma volume. In the remaining twelve cases, the decrease in basal metabolic rate was accompanied by a decrease in plasma volume of 2.2 to 29.5 per cent (average 12.0 per cent). Fifteen of the seventeen cases showed a cell volume decrease, the average change being 13.8 per cent of the original cell volume. The data of Gibson and Harris therefore agree with the subnormal hematocrit values we have found. The increased serum globulin concentration may be due to hemoconcentration but this is not yet proved, particularly in the light of the data of Gibson and Harris, which show an increase in plasma volume with decreased basal metabolic rate in one-third of their human subjects.

The data of Table I as well as those previously reported (1) show that the electrophoretic and salt fractionation methods agree in defining the direction of the changes in concentration of serum albumin and globulin. It may be noted, however, that the ratio of albumin to globulin as determined by electrophoresis is consistently higher than the ratio obtained by the salt fractionation method. It is recognized that the albumin to globulin ratio as determined by electrophoresis is dependent on both the protein concentration and the concentration and nature of the buffer ions used. It has been shown, however, that the sodium phosphate-saline buffer and the protein concentration employed in these experiments yield a relation which reduces almost to a minimum the error obtained with buffers of lower ionic strength and protein of higher concentration (8).

Moreover, the possibility that a part of the electrophoretic albumin is precipitated along with the globulin by the high salt concentration used for the chemical separation has previously been suggested (1). To investigate this possibility a sample of pooled rat serum was fractionated into "albumin" and "globulin" fractions by addition to 19 volumes of 21 per cent sodium sulfite. The "albumin" filtrate was concentrated by pressure dialysis and then dialyzed against large volumes of the pH 7.4 sodium phosphate buffer described above.

The precipitated globulin was washed with a small volume of fresh solvent, centrifuged down to form a compact pellet, and the supernatant decanted. The precipitate was then dissolved in water and an aliquot of the solution dialyzed against large volumes of the pH 7.4 buffer. The remainder of the "globulin" solution was reprecipitated by adding it to 19 volumes of 21 per cent sodium sulfite and, after repetition of the washing procedure, another aliquot was removed and dialyzed. The remainder was precipitated for the third time and this precipitate dissolved and dialyzed. The four resulting solutions, namely that of "albumin" and of "globulins" precipitated one, two, and three times respectively, were analyzed electrophoretically.

Patterns of the unfractionated serum and the "albumin" filtrate are reproduced in Fig. 2. An appreciable amount of globulin has remained unprecipitated (Fig. 2, *B*). Also the "globulin" precipitate contained a considerable quantity of electrophoretic albumin, as is indicated by the patterns of Fig. 3. A comparable result was obtained with another sample of pooled rat serum treated in a similar manner.

The fact that the attempt to separate the electrophoretic albumin from the precipitated "globulins" by repeated sodium sulfite precipitations did not succeed suggests that this albumin differs from the main bulk of the serum albumin. Supporting evidence is afforded by its mobility ($u = -4.0 \times 10^{-5}$ sq. cm. per volt per second) which is definitely less than that ($u = -4.6 \times 10^{-5}$ sq. cm. per volt per second) of the albumin not precipitated by sodium sulfite.[3] These results suggested to us the possibility that the fraction having the lower mobility might be the high carbohydrate-containing fraction isolated by McMeekin (9). A carbohydrate analysis[4] was, therefore, attempted on the small amount of material at our disposal. The analyses were made by a quantitative modification of the Molisch reaction depending on comparison of the color developed by the unknown against the color developed by glucose standards. Because the color produced by the proteins was not identical with that of the glucose standard, the absolute values for carbohydrate content cannot be stated with accuracy. The analysis did definitely show, however, that the albumin fraction precipitated by the sodium sulfite contained about 2.5 times as much carbohydrate as does the albumin which is not precipitated by sodium sulfite.

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[3] The mobilities of all the globulin fractions were increased by each successive precipitation and the third precipitate gave a distorted pattern with little or no protein having the mobility of γ-globulin. The mobility of the fastest component (presumably albumin) did not change appreciably with reprecipitation.

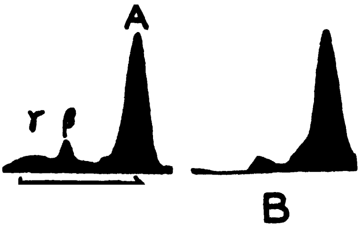

Fig. 2. (*A*) Pooled unfractionated serum of normal rats. (*B*) 20 per cent sodium sulfite filtrate of the same serum. Buffer, as in Fig. 1.

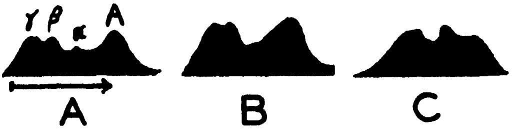

Fig. 3. 20 per cent sodium sulfite precipitate from normal rat serum after (*A*) first precipitation, (*B*) reprecipitation, and (*C*) second reprecipitation. Buffer, as in Fig. 1.

The actual figures obtained, 4.3 and 1.8 per cent carbohydrate, respectively, for the precipitated and non-precipitated fractions, indicate that the precipitated fraction is indeed similar to the albumin fraction (5.5 per cent carbohydrate) isolated by McMeekin (9) from horse serum.

It was also of interest to see whether the electrophoretic albumin in the precipitate was similar to the α_1-globulin fraction described by Longsworth (10) and found in sera from normal and hypophysectomized rats (1) when the analyses were made in sodium diethylbarbiturate buffer at pH 8.6. Whole rat serum and the filtrate fraction were analyzed in this buffer. As may be seen in Fig. 4, the ratio of the two components was unchanged after precipitation and removal of the precipitate. The mobilities of all of the fractions were, however, appreciably increased by the sodium sulfite. It is evident, therefore, that the second component in the patterns obtained at pH 8.6 is not selectively precipitated with the globulins by 20 per cent sodium sulfite, and it is probable that this is a third albumin fraction (the first being the fastest moving component in the patterns obtained at pH 8.6 and the second being in the sodium sulfite precipitate), since by the criteria of salt fractionation and electrophoresis at lower pH it is albumin. At present it is impossible to say, however, whether the fractions are native or are produced by the procedures employed.

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[4] We are greatly indebted to Miss Marion Blanchard for these analyses.

An attempt was made to use ultracentrifugal data as a criterion for establishing whether the various substances were albumins or globulins, but unfortunately all fractions, even though they were clear solutions, yielded sedimentation constants which indicated aggregation. Comparison of serum protein fractions obtained by other chemical means known not to affect the physical properties of the protein appreciably is in progress and will be reported later.

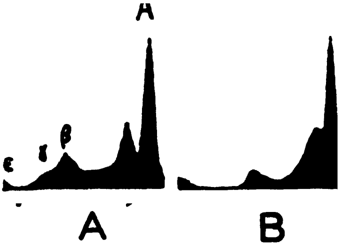

FIG 4. Pooled unfractionated serum of (*A*) normal rats. (*B*) 20 per cent sodium sulfite filtrate. Buffer, sodium diethylbarbiturate at pH 8.6.

The authors are indebted to Miss Helen Sikorski and Miss Dorothy Wangerin for technical assistance.

SUMMARY

The protein component usually designated as α-globulin was found in definite quantities in only four of twenty sera of normal rats. In contrast, this component was found in the serum of every hypothyroid rat examined, whether the hypothyroidism was due to thyroidectomy or to thiouracil feeding.

The globulins precipitated from rat serum by 20 per cent sodium sulfite contained a component having an electrophoretic mobility of serum albumin. This "albumin" was not separated from the globulins by repeated reprecipitation with sodium sulfite.

BIBLIOGRAPHY

1. Moore, D. H., Levin, L , and Leathem, J. H., *J. Biol Chem.*, **153**, 349 (1944).
2. Levin, L., and Leathem, J. H., *Am. J. Physiol.*, **136**, 306 (1942).
3. MacKenzie, C. G., and MacKenzie, J. B., *Endocrinology*, **32**, 185 (1943). Astwood, E. B., Sullivan, J., Bissell, A., and Tyslowitz, R., *Endocrinology*, **32**, 210 (1943). Astwood, E. B., *J. Pharmacol. and Exp. Therap.*, **78**, 79 (1943).
4. Levin, L., *Am. J. Physiol* , **138**, 258 (1943).
5. Ross, V., Moore, D. H , and Miller, E. G., Jr., *J. Biol. Chem.*, **144**, 667 (1942).
6. Campbell, W. R., and Hanna, M. I., *J. Biol. Chem.*, **119**, 15 (1937).
7. Gibson, J. G., and Harris, A. U., *J. Clin. Invest.*, **18**, 59 (1939).
8. Svensson, H., *Ark. Kemi, Mineral. o. Geol.*, **17**, No. 14 (1943).
9. McMeekin, T. L., *J Am Chem. Soc.*, **62**, 3393 (1940).
10. Longsworth, L. G., *Chem. Rev.*, **30**, 323 (1942).

STUDIES ON THE ENZYME WHICH PRODUCES THE STREPTOCOCCUS LACTIS R-STIMULATING FACTOR FROM INACTIVE PRECURSOR SUBSTANCE IN YEAST*

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Totter, Mims, and Day (1) showed that yeast contains a substance (or substances) which in itself is inactive, but which can easily be changed into the *Streptococcus lactis* R-stimulating factor by incubation with fresh rat liver. Mims, Totter, and Day (2) described a method for the determination of this potential factor in biological materials. The method is based on the incubation of the material to be examined with a slightly purified extract of fresh rat liver, followed by microbiological assay with *Streptococcus lactis* R as the test organism.

Binkley *et al.* (3) have recently announced the concentration of the chick antianemia factor from yeast, and reported that it is relatively inert in stimulating the growth of *Lactobacillus casei*, but becomes highly active after enzymatic digestion. They applied the term "vitamin B_c conjugate" to this substance. Crystalline vitamin B_c was isolated from digests of the conjugate. The source of their enzyme, however, was not disclosed.

The object of the present study was to purify and to investigate the properties of the enzyme which is capable of transforming inactive precursor substances into the *Streptococcus lactis* R-stimulating factor.

Methods

A commercial yeast extract preparation (Difco)[1] was used as a crude substrate for the enzymatic reaction (2). The product, or products, of the reaction was determined by microbiological assay with *Streptococcus lactis* R, according to the procedure described by Mitchell and Snell (4) for the determination of folic acid.

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[1] Bacto-yeast extract, Difco Laboratories, Inc., Detroit A single-batch (Control 365855) of this extract was used throughout the work reported here It contained 200 γ of potential *Streptococcus lactis* R-stimulating factor per gm It is believed, however, that the enzyme can be standardized on any other batch of this brand of yeast extract, provided that an amount is used which furnishes a suitable amount of the precursor substance (see Fig. 1).

In order to express the activity of the enzyme in a quantitative way, a provisional unit was defined as follows: 1 unit represents the amount of enzyme which will produce 1 γ of the factor (as folic acid of potency 40,000) per hour when incubated at 37° with 200 mg. of the yeast extract (Difco);[1] total volume 11 cc., incubation 4 hours.

Most of the actual determinations of enzymatic activity of the more highly purified extracts were done on quantities one-tenth of those stated above; only 1 cc. of substrate solution (containing 20 mg. of yeast extract) and 0.1 cc. of enzyme solution were used. Fairly good proportionality was found between the amount of factor formed and the amount of enzyme used when the latter was between 0.05 and 0.5 unit (Table I).

The amount of enzyme in the tissues studied was determined as follows: A sample of fresh tissue was weighed, thoroughly ground with sand, extracted with 5 volumes of 0.1 M phosphate buffer, pH 7, and centrifuged. An aliquot of the supernatant was incubated with the excess of yeast extract as previously described. Usually the reaction was allowed to proceed for a period of 4 hours, and was then stopped by placing the tube in a boiling water bath for a period of 5 minutes. The mixture was centrifuged and the supernatant was diluted to a convenient volume. Aliquots were taken for the determination of *Streptococcus lactis* R growth-stimulating factor. Corrections for the blanks, due to the content of preformed factor, were subtracted.

TABLE I

Recovery of Enzyme in Different Dilutions

The enzyme solution contained 120 units per cc.* Each tube contained 0.1 cc. of the diluted enzyme and 1 cc. of 2 per cent solution of yeast extract (Difco) in phosphate buffer.

Dilution	Calculated	Found
	unit	*unit*
1 : 24	0.5	0.45
1 : 48	0.25	0.22
1 : 120	0.1	0.105
1 : 240	0.05	0.07

* Average of several determinations on the undiluted solution.

The potency of the enzyme in a given preparation is expressed as the number of units per mg. of protein, which was determined by the method of Robinson and Hogden (5).

EXPERIMENTAL

The distribution of the enzyme was first investigated in different organs of the rat. The results are shown in Table II. It is obvious that the enzyme is widely distributed throughout the rat's body. Pancreas, brain, intestinal mucosa, and bone showed much higher values than liver, under the conditions described above.

For the purpose of this study, however, it was inconvenient to depend upon rat tissue as a source of enzyme. Organs of several other animals were therefore tested (Table III). Chicken pancreas was chosen because it was rich enough in enzyme and rather easy to obtain.

TABLE II

*Distribution of Enzyme in Rat Organs and Tissues**

Organ	Units of enzyme per gm. protein in tissue extract			
	Experiment 1	Experiment 2	Experiment 3	Experiment 4
Brain	288	180	220	328
Pancreas	300	46	108	227
Bone	198	190		
Intestinal mucosa	100	97		
Kidney	178	52	120	
Spleen	150	85		
Muscle	92	38		
Heart	61	49		
Liver	21	15	16	

* In the experiments on which these data are based the concentration of the crude substrate (yeast extract, Difco) was 20 mg. per cc. Different relative values have been obtained for certain tissues by altering the amount of substrate.

TABLE III

Distribution of Enzyme in Organs and Tissues of Different Species

Organ	Less than 50 units per gm. protein	Between 50-100 units per gm. protein	Organ	Less than 50 units per gm. protein	Between 50-100 units per gm. protein	Over 100 units per gm. protein
Dog pancreas	+		Rabbit pancreas	+		
Hog brain	+		" bone marrow	+		
Beef brain	+		Chicken brain		+	
" muscle	+		" pancreas			+
" pancreas	+		" intestinal mucosa			+
" bone marrow	+		Chicken liver			+
Rabbit brain		+	" kidney		+	
" muscle	+		" spleen	+		

Preparation of Enzyme

Extraction—Approximately 0.5 kilo of fresh chicken pancreas was collected at a poultry market. It was first ground in a meat grinder, and then in a mortar with sand. It was extracted with approximately 3 volumes (1.5 liters) of 0.1 M phosphate buffer, pH 7, and centrifuged. The fat collecting on the top of the tubes was discarded. The brownish extract usually contained 4000 units of the enzyme per kilo of fresh pancreas and the potency was about 0.13 unit per mg. of protein.

Purification with Tricalcium Phosphate Gel—The crude extract was treated with an equal volume of approximately 0.1 M tricalcium phosphate suspension. The adsorption complex was centrifuged down and discarded. The opalescent, yellowish supernatant was collected. No loss of activity occurred at this stage, whereas the amount of total protein was reduced by one-half.

Precipitation with Alcohol—The enzyme solution was chilled to 10° and was treated with an equal volume of ice-cold 95 per cent alcohol. The mixture was left in an ice bath for 1 hour and then centrifuged. The supernatant was discarded. The precipitate was thoroughly mixed with about 120 cc. of phosphate buffer[2] and the denatured protein then centrifuged off and discarded. The supernatant was collected; it contained above 80 per cent of the original activity and had a potency of about 7 units per mg. of protein.

Salting-Out with Sodium Sulfate—The enzyme solution at this stage was too dilute to be convenient for further purification. In order to reduce the volume it was saturated with anhydrous sodium sulfate at 37° (45 gm. per 100 cc. of the enzyme solution). It was then placed in the refrigerator and allowed to crystallize overnight. The slightly cloudy supernatant was decanted, the crystals of decahydrous sodium sulfate were washed with a little of the ice-cold buffer, and the washings added to the decanted supernatant. The crystals were discarded. This procedure reduced the volume to about one-half of the original.

Three or four preparations were carried out up to this stage and were then combined. The volume was further reduced by repeated saturation with sodium sulfate as described above. Finally the enzyme solution was evaporated in front of a fan at room temperature for a period of 2 to 4 hours. When a concentration of protein of at least 5 mg. per cc. was achieved, the liquid was again saturated with sodium sulfate at 37° and was centrifuged. During the centrifugation the excess salt was thrown down and the insoluble enzyme collected on the surface of the liquid. It was separated and resuspended in a small amount of buffer solution. A significant increase in potency was observed at this stage. It was mainly due to the removal of nitrogen-containing substances of relatively low molecular weight, which gave a strong biuret reaction, although giving only slight precipitate with trichloroacetic acid.

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[2] In several preparations phosphate buffer was replaced by borate buffer without any alteration of the course of purification.

Second Purification with Tricalcium Phosphate Gel and Precipitation with Alcohol—The excess sodium sulfate was removed by 24 hours dialysis against phosphate buffer in the cold. The enzyme solution was then treated with $Ca_3(PO_4)_2$ gel and was precipitated with alcohol as described above. The alcohol precipitate was extracted with about 20 cc. of buffer and then centrifuged. The extract was collected and saturated with sodium sulfate. At this stage the enzyme could be preserved in the refrigerator for at least 3 weeks without loss. It still contained about 70 per cent of the original activity and had an average potency of 200 units per mg. of protein. In one case a potency of 500 units per mg. of protein was obtained. Table IV summarizes the steps of purification.

Table IV

Summary of Potency and Yield of Enzyme after Various Steps in Purification Procedure

Steps in purification	Potency per mg protein	Yield	
		From kilo fresh tissue	Per cent recovery*
	units	*units*	
Crude extract of chicken pancreas	0.13	4100	100
After treatment with Ca phosphate gel	0.25	4200	103
After pptn. with alcohol	7	3500	86
After concentration and pptn. with Na_2SO_4	20	3400	83
After final pptn. with Na_2SO_4	200	2800	70

* Expressed as per cent of the content of enzyme in the crude extract of chicken pancreas.

Conditions of Reaction and Some Properties of Partially Purified Chicken Pancreas Enzyme

In spite of the fact that the exact chemical nature of neither the substrate nor the product of the reaction is known, it has been possible to study some of the conditions of the reaction by following the liberation of the bacterial growth-stimulating factor.

The influence of the concentration of the crude substrate was first investigated. The results are shown in Fig. 1. In order to secure maximal velocity of the reaction under the conditions of the experiment, at least 10 mg. of yeast extract (Difco) were required. No decrease in the quantity of reaction product was observed with 4 times this amount of substrate. The effect of varying the concentration of the enzyme is shown in Fig. 2. In order to carry the reaction to completion a very large quantity of the enzyme was required.

The effect of pH was also studied. With the crude extracts the optimum appeared to be at pH 7 and this pH was therefore chosen for the testing of activity. With more highly purified enzyme preparations the optimal activity was at pH 8. The difference in activity at pH 7 and 8 was, however, comparatively small. Fig. 3 shows the results of an experiment in which a large excess of substrate was present, and in which the reaction was stopped when less than 10 per cent of the substrate was utilized. The influence of pH was also investigated in another set of experiments in which a study of the time-activity relation was the main object. Fig. 4 presents some of the results of this experiment. Very little difference was found between the rate of the reaction at pH 7.0 and 7.9, except that the results for the latter pH were less erratic. The reaction at pH 5 started with a lower, but still comparatively high, velocity. Complete utilization of the substrate apparently did not take place at pH 5, probably because of the destruction of enzyme.

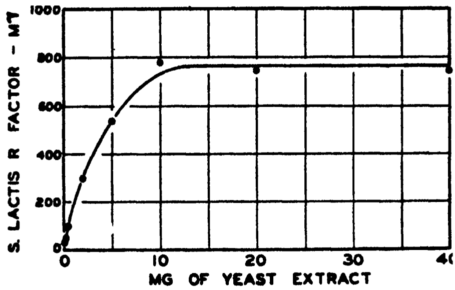

FIG. 1. Rate of formation of *Streptococcus lactis* R-stimulating factor as a function of the concentration of the substrate. Each tube contained 0.1 cc. of enzyme solution, equivalent to 1.2 units, and 1 cc. of phosphate buffer containing from 0.1 to 40 mg. of yeast extract (Difco). Incubation 15 hours.

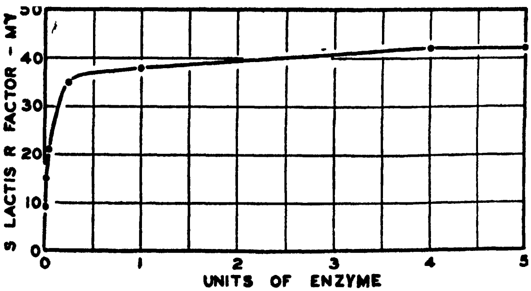

FIG. 2. Rate of formation of *Streptococcus lactis* R-stimulating factor as a function of the enzyme concentration. Each tube contained 200 γ of yeast extract (Difco) and amounts of enzyme from 0.01 to 5 units. Total volume 1.1 cc.; incubation 15 hours.

The activity of the enzyme preparation was tried on a concentrate of vitamin B_c conjugate (3), kindly supplied by Dr. J. J. Pfiffner of Parke, Davis and Company, and stated to contain 1.8 γ of vitamin B_c in the free state and 36 γ of vitamin B_c, including both free and combined forms, per cc. (as determined by the growth of *Lactobacillus casei*). The enzyme was found to be active upon this substrate, liberating even more than

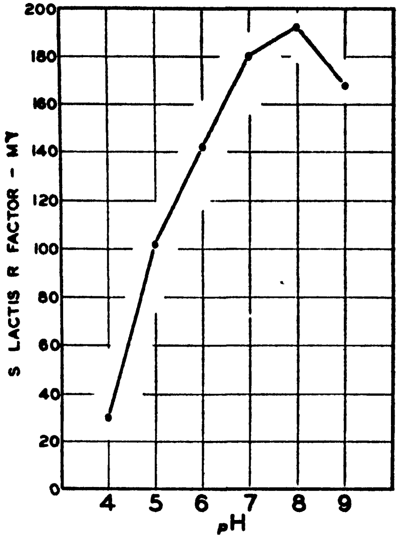

FIG. 3. Rate of formation of *Streptococcus lactis* R-stimulating factor as a function of pH. Each tube contained 0 6 unit of enzyme and 20 mg of yeast extract (Difco). Total volume 1.1 cc.; incubation 7 hours. pH 4 and 5, acetate buffer; pH 6 and 7, phosphate buffer; pH 8 and 9, borate buffer.

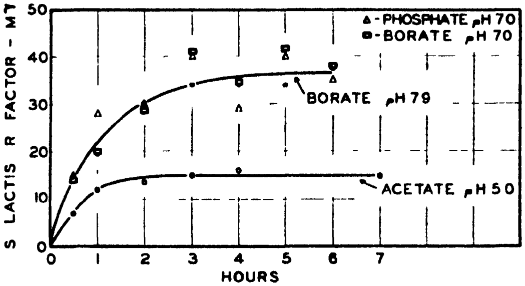

FIG. 4. Time-activity curves of the purified enzyme preparation. Each tube contained 0.6 unit of enzyme and 200 γ of yeast extract (Difco) Total volume 1.1 cc.

stated amounts of the growth-stimulating factor (as determined by the growth of *Streptococcus lactis* R) when checked against a standard crystalline preparation of free vitamin B_c, also kindly supplied by Dr. Pfiffner. Since the same preparation of purified enzyme liberated the *Streptococcus* *lactis* R-stimulating factor from both a concentrate of vitamin B_c conjugate and the yeast extract (Difco), it seems reasonable to conclude that the actual substrates in both cases must have been similar and contained at least one identical group.

TABLE V

Effect of Exposure to Heat on Activity of Enzyme

The figures represent the per cent of the original activity remaining after heat treatment. Each tube contained 0.5 cc. of the enzyme solution, equivalent to 3 units. After exposure to the indicated temperatures the tubes were cooled, 5 cc. of 2 per cent yeast extract (Difco) solution in strong buffer were added to each tube, and they were incubated 4 hours.

Temperature	pH 5; exposure 5 min	pH 7		pH 9; exposure 5 min.
		Exposure 1 min.	Exposure 5 min.	
°C.	*per cent*	*per cent*	*per cent*	*per cent*
30	75	100	100	100
45		80		
50		60		
60	7	50	21	10
70		26		
80	2	18	6	2
100	2	10	3	2

TABLE VI

Effect of Digestion with Trypsin on Activity of Enzyme

Each tube contained 0.1 cc. of the enzyme solution, equivalent to 1.8 units, and 1 cc. of trypsin solution in borate buffer, pH 8.0. All tubes were incubated 7 hours at 37°, after which 1 cc. of 4 per cent yeast extract (Difco) solution was added to each tube and they were then further incubated for 4 hours at 37°. The reaction was stopped by boiling.

Crystalline trypsin	Activity recovered
mg.	*per cent of original enzyme*
0	100
0.01	83
0.1	83
1.0	33
6.0	22

The enzyme was found to be precipitated with 75 per cent acetone at neutral pH, but some loss of activity occurred even at 0°. The enzyme was poorly adsorbed at pH values between 5 and 7 on either calcium phosphate gel or on alumina Cγ, and large quantities of adsorbents were required. Once adsorbed it could be rather easily eluted with 0.1 M borate buffer, pH 9.

Dialysis against water for 12 hours at refrigerator temperature resulted in 80 per cent loss of activity.

The velocity of the reaction was not decreased when incubation with the enzyme was carried out in an atmosphere of pure nitrogen, which indicates that free oxygen is not essential for the reaction.

The enzyme was easily inactivated by heat. The resistance to elevated temperatures varied at different pH values; the results are shown in Table V.

The enzyme was very resistant to the action of crystalline trypsin, prepared according to the method of Kunitz and Northrop (6). The results are shown in Table VI.

SUMMARY

A method is described for the quantitative determination of the enzyme which produces the *Streptococcus lactis* R growth-stimulating factor from inactive precursor substances in yeast.

The distribution of the enzyme in several organs of the rat, and in a few organs of the dog, hog, cow, rabbit, and chicken, is reported.

A method leading to the partial purification of this enzyme is described, which involves treatment with calcium phosphate gel, precipitation with alcohol, and repeated concentration of the solution and salting-out with sodium sulfate By this method a preparation with an average potency of 200 units per mg. of protein, with a yield of 70 per cent, was obtained from chicken pancreas.

The conditions of the enzymatic reactions have been studied, including enzyme-substrate and time-activity relations. The enzyme exhibits optimal activity between pH 7 and 8, it is rapidly inactivated by exposure to temperatures above 45°, and it is relatively stable to the action of crystalline trypsin.

BIBLIOGRAPHY

1 Totter, J R , Mims, V , and Day, P L , *Science*, **100**, 223 (1944)
2 Mims, V , Totter, J R , and Day, P L , *J Biol Chem* , **155**, 401 (1944)
3 Binkley, S B , Bird, O D , Bloom, E S , Brown, R A , Calkins, D G , Campbell, C J , Emmett, A D , and Pfiffner, J J , *Science*, **100**, 36 (1944)
4 Mitchell, H K , and Snell, E E , *Univ Texas Pub* , *No 4137*, 36 (1941)
5 Robinson, H W , and Hogden, C G , *J Biol Chem* , **135**, 727 (1940)
6 Kunitz, M , and Northrop, J H , *J Gen Physiol* , **19**, 991 (1936)

THE SPECIFICITY OF THE LEUCINE, ISOLEUCINE, AND VALINE REQUIREMENTS OF LACTOBACILLUS ARABINOSUS 17-5

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Data obtained previously (1) suggested partial activity of $d(-)$-leucine in supplying the leucine requirement of *Lactobacillus arabinosus.* Further studies reported in this paper have confirmed this observation. We have also tested the activity of the isomers of isoleucine, and compounds related to leucine, isoleucine, and valine in replacing these amino acids. Data upon the activity of the isomers of the amino acids are needed before the synthetic racemic acids can be justifiably used as standards in microbiological assays, and may also throw some light upon amino acid metabolism.

EXPERIMENTAL

The basal medium and techniques used in these assays were the same as previously described (2), except that glycine was added to supply 0.5 mg. per tube. The rate of growth was followed in these studies by determining the turbidity of the tubes at convenient intervals during the growth period. Characteristic differences in the time that growth is initiated and in the rate of growth furnish evidence that the activity is due to the compound under investigation and not to contamination of the corresponding amino acid. Lactic acid production was also measured at the end of the growth period by titration with 0.05 N alkali. Titrations were made at times which varied from 67 to as long as 150 hours after seeding, depending upon the rate of growth. Activity of the material was calculated either from turbidity readings or from lactic acid production. Several separate assays at a series of levels were made for each compound studied.

We are indebted to several people who generously contributed samples of various compounds for this investigation. We wish to thank Dr. W. C. Rose for samples of $d(-)$-valine, sodium α-ketoisocaproate, $l(+)$-isoleucine, $d(-)$-isoleucine, $l(+)$-alloisoleucine, and $d(-)$-alloisoleucine; Dr. David Bonner for samples of tertiary *dl*-leucine, calcium *dl*-α-hydroxyisovalerate, calcium *dl*-α-hydroxy-β-methyl-*n*-valerate, and calcium *dl*-α-hydroxyisocaproate (3); Dr. Sarah Ratner for two samples of $d(-)$-leucine; and Dr. Halvor N. Christensen for acetyl-$l(+)$-leucine, acetyl-*dl*-valine,

acetyl-*dl*-isoleucine, and the benzoyl derivatives of *l*(+)-valine, *l*(+)-valyl-*l*(+)-valine, *l*(+)-valyl-*d*(−)-valine, *d*(−)-valyl-*d*(−)-valine, and *d*(−)-valyl-*l*(+)-valine (4).

Results

Leucine—The approximate activity of the compounds tested for their ability to replace leucine is shown in Table I. In several instances absolute figures are not given, since the activity depends upon the amount of material added or upon the time that the assay was completed. In particular, with *d*(−)-leucine growth was very slow and the tubes continued to show increases in turbidity even after 5 days. Maximum growth was obtained with the natural isomer in about 60 hours (Fig. 1). Also, unknown circumstances probably relating to the condition and age of the culture or to slight variations in the media appear to have an effect, since consistent results on successive assays are not always obtained, although the *d*(−)-leucine always showed some activity. It may be noted that growth is initiated at approximately the same time in tubes containing *l*- or *d*-leucine. This is probably due to small amounts of the natural isomer in the *d*(−)-

TABLE I

*Activity of Various Compounds in Replacing Leucine, Isoleucine, and Valine**

Leucine		Isoleucine		Valine	
Compound tested	Approximate activity	Compound tested	Approximate activity	Compound tested	Approximate activity
	per cent		*per cent*		*per cent*
l(+)-Leucine	100	*l*(+)-Isoleucine	100	*l*(+)-Valine	100
d(−)-Leucine	20–60	*d*(−)-Isoleucine	0	*d*(−)-Valine	0
Na α-ketoisocaproate	50	*l*(+)-Alloisoleucine	25–50	α-Ketoisovaleric acid	Active
Ca *dl*-α-hydroxyisocaproate	0	*d*(−)-Alloisoleucine	0	Ca *dl*-α-hydroxyisovalerate	0–75
dl-Norleucine	0	Acetyl-*dl*-isoleucine	0	Acetyl-*l*(+)-valine	0
Tertiary *dl*-leucine	0	*dl*(−)-Norleucine	0	Benzoyl-*l*(+)-valine	0
Acetyl-*dl*-leucine	30–60	Tertiary *dl*(−)-leucine	0	Benzoyl-*l*(+)-valyl-*l*(+)-valine	0
dl-Leucylglycine	50	α-Keto-β-methyl-*n*-valerate	Active	Benzoyl-*l*(+)-valyl-*d*(−)-valine	0
Glycyl-*l*(+)-leucine	100	Ca *dl*-α-hydroxy-β-methyl-*n*-valerate	80–100	Benzoyl-*d*(−)-valyl-*d*(−)-valine	0
				Benzoyl-*d*(−)-valyl-*l*(+)-valine	0

* On a molecular basis.

leucine sample. This sample of $d(-)$-leucine contained 6.7 per cent of the l isomer as determined by the isotopic dilution method.[1]

It is of interest that the corresponding keto acid is only 50 per cent as active as $l(+)$-leucine. Growth was initiated at the same time as for $l(+)$-leucine and completed in approximately the same time. Thus this figure should represent true activity. In order to prevent destruction of the α-keto acid in these assays, it was added in sterile solution after the remainder of the medium had been autoclaved. About 20 per cent was destroyed if this precaution was not observed.

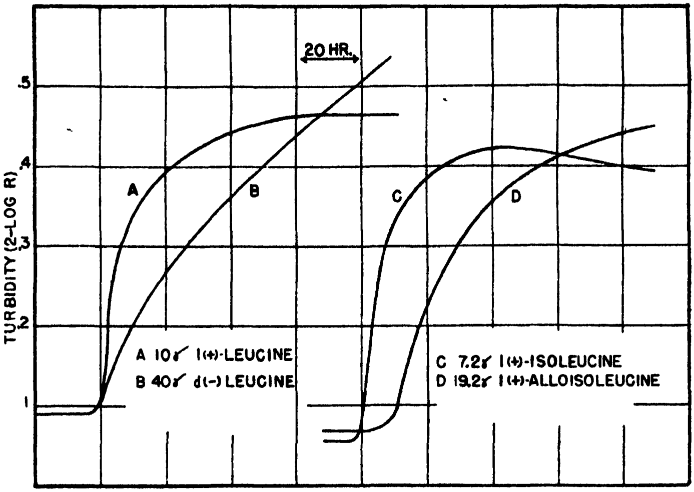

FIG. 1 Growth curves showing the relative effectiveness of $d(-)$-leucine in replacing $l(+)$-leucine, and of $l(+)$-alloisoleucine in replacing $l(+)$-isoleucine.

dl-Leucyglycine and glycyl-$l(+)$-leucine showed full activity on the basis of their $l(+)$-leucine content, and the rate of growth was similar to that obtained with leucine. However, acetylleucine showed erratic results, and only occasional tubes reached the level of growth expected. No activity for α-hydroxyisocaproic acid, norleucine, or tertiary leucine was detected.

Isoleucine—Of the optical isomers of this amino acid, both of the l acids were active, but the rate of growth with $l(+)$-alloisoleucine is much less than with the natural amino acid. As shown in Fig. 1, there is also a lag before growth begins. The maximum activity of this isomer which has been obtained was 50 per cent after 115 hours of incubation. The difference

[1] Dr. Sarah Ratner, personal communication.

in the characteristics of the growth curves appears to rule out the possibility that the activity was due to isoleucine contamination.

Treatment of *d*(−)-isoleucine with a *d*-amino acid oxidase preparation[2] yielded a preparation with much more activity than appropriate controls, thus demonstrating the activity of the corresponding α-keto acid. The great activity of the corresponding α-hydroxy acid is of interest in view of the failure of α-hydroxyisocaproic acid to show leucine activity. Although growth begins somewhat later, after 80 to 100 hours the turbidity or acid production is about the same as with *l*(+)-isoleucine. It thus appears that there is practically complete conversion to isoleucine, whereas only half of the α-ketoisocaproic acid is converted to leucine.

No activity for the acetyl derivative could be shown. In recovery experiments in which small known amounts of isoleucine were added to tubes containing acetylisoleucine, visible growth developed somewhat later than in standard tubes, but the total amount of growth at 70 hours was unaffected by the addition of acetylisoleucine.

Valine—α-Ketoisovaleric acid was shown to replace valine by the increase in activity after incubation of *d*(−)-valine with *d*-amino acid oxidase. Of the other compounds tested, only α-hydroxyisovaleric acid proved active. The results were erratic. Growth usually did not occur if less than 20 γ were added per tube. At higher levels, if growth was initiated, it sometimes exceeded that of *dl*-valine, indicating more than 50 per cent conversion to valine. The addition of small known amounts of valine did not overcome the great variation in the ability of the cultures in certain tubes to use α-hydroxyisovaleric acid.

DISCUSSION

Although it has been generally concluded that only the naturally occurring enantiomorphs of the amino acids are active for *Lactobacilli* (5), it appears that in several cases the test has not been sufficiently rigorous to prove this point. With both *l*(+)-alloisoleucine and *d*(−)-leucine, the activity can only be shown with surety if an incubation period longer than 72 hours and the isolated unnatural enantiomorph are used. From our data it can be seen that the activity of *d*(−)-leucine could be detected only with difficulty in a comparison of *l*- and *dl*-leucine, especially if the incubation period were the usual 72 hours. Probably not more than 10 per cent activity of *d*(−)-leucine could be found, an amount within the usual error of an assay. In a given series of tubes at different concentrations, increasing inaccuracy may be found on either side of an optimum range, but it is doubtful whether one may justifiably discard all other points except for a single one which appears optimum, as has been done by Kuiken *et al.* (6). However, it is equally clear that the error occasioned by the use of *dl*-amino acids as standards in ordinary assays will be small.

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[2] This preparation was kindly supplied by Dr. H. D. Hoberman, Department of Comparative Pathology and Tropical Medicine, Harvard Medical School.

The ability of *Lactobacillus arabinosus* to utilize *d*(−)-leucine is of interest in view of the presence of large amounts of *d*(−)-leucine in the bacterial product, gramicidin.[3] The fact that only 50 per cent activity for α-ketoisocaproic acid could be shown may suggest an indiscriminate synthesis of either enantiomorph from the keto acid.

The variation in the activity of the various α-hydroxy acids is of interest. These three amino acids can be arranged in order of decreasing availability of the corresponding hydroxy acid. α-Hydroxy-β-methylvaleric acid is practically the equivalent of isoleucine; α-hydroxyisovaleric acid is used with difficulty in the place of valine; and α-hydroxyisocaproic acid will not replace leucine. If the conversion to the corresponding amino acid is through the intermediate keto acid, it is apparent that there is also a variation in the utilization of these, since α-ketoisocaproic acid is less effective in replacing leucine than is *dl*-α-hydroxy-β-methylvaleric acid in replacing isoleucine.

The difference in the utilization of the acetyl derivatives also indicates the great specificity of the enzymatic make-up of the organism. Acetylleucine is used with relative ease, but acetylisoleucine or acetylvaline is completely unavailable. The failure in utilization is not due to a quantitative deficiency of the appropriate enzyme, since these derivatives show no activity in recovery experiments. Such studies have also shown that the acetyl derivatives are not toxic at the levels tested.

SUMMARY

The isomers of leucine, isoleucine, and valine and various derivatives of these amino acids were tested for their ability to replace these amino acids in the nutrition of *Lactobacillus arabinosus*. In the absence of *l*(+)-leucine, *d*(−)-leucine allows some growth, although the rate is much below normal. Similarly, *l*(+)-alloisoleucine is capable of partially replacing isoleucine. Isoleucine is effectively replaced, valine partially, and leucine not at all by the corresponding α-hydroxy acids. Of the acetyl derivatives only acetylleucine was active. All three of the α-keto acids are active, although these have not been measured quantitatively except for α-ketoisocaproic acid, which is 50 per cent as active as *l*(+)-leucine.

Thanks are due to Miss Eunice D. Wardwell and Miss M. Lois Butler for technical assistance.

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[3] Repeated subculture in a medium containing *d*(−)-leucine failed to improve the utilization of this amino acid.

BIBLIOGRAPHY

1. Hegsted, D. M., and Wardwell, E. D., *J. Biol. Chem.*, **153**, 167 (1944).
2. Hegsted, D. M., *J. Biol. Chem.*, **152**, 193 (1944).
3. Bonner, D., Tatum, E. L., and Beadle, G. W., *Arch. Biochem.*, **3**, 71 (1943).
4. Christensen, H. N., *J. Biol. Chem.*, **154**, 427 (1944).
5. Stokes, J. L., and Gunness, M., *J. Biol. Chem.*, **154**, 715 (1944).
6. Kuiken, K. A., Norman, W. H., Lyman, C. A., Hale, F., and Blotter, L., *J. Biol. Chem.*, **151**, 615 (1943).

LETTERS TO THE EDITORS

A STUDY OF THE INTERMEDIATES OF ACETOACETATE OXIDATION WITH ISOTOPIC CARBON

Sirs:

The hypothesis proposed by Breusch[1] and by Wieland and Rosenthal[2] that acetoacetate is metabolized via intermediates of the tricarboxylic acid cycle has been studied with the use of acetoacetic acid marked with C^{13} in the carboxyl and β-carbon positions (*i e.* $CH_3C^{13}OCH_2C^{13}OOH$).

We have found that citrate, α-ketoglutarate, succinate, fumarate, malate, or oxalacetate, when added in proper concentrations to homogenates of guinea pig kidney cortex, stimulates the disappearance of acetoacetate. To test whether a part of this metabolism proceeds by oxidative reactions in which the intermediates of the tricarboxylic acid cycle are formed from acetoacetate, the following experiments were performed. To 10 gm. of guinea pig kidney cortex homogenized in 65 ml. of Krebs' saline were added sodium bicarbonate, sodium succinate, and isotopic sodium acetoacetate so that the final concentrations of these substances in a total volume of 100 ml. were 40, 15, and 4 mM per liter respectively. The homogenate was incubated aerobically at 38° for 60 minutes at pH 7.4. After deproteinization, fumaric acid was isolated as the di-*p*-nitrobenzylthiuronium derivative.

In a second experiment the oxidation of isotopic acetoacetate was stimulated by the addition of sodium α-ketoglutarate (15 mM per liter) and after 40 minutes the residual α-ketoglutaric acid was isolated as the semicarbazone.

Experiment No	Acetoacetate metabolized	C^{13} concentration, atoms per cent excess		
		Initial acetoacetate	Final fumarate	Final α-ketoglutarate
	mM			
1, A	0.25	3.80	0.22	
1, B	0.34	3.98	0.31	
2	0.32	3.98		0.50

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[1] Breusch, F. L., *Science*, **97**, 480 (1943).

[2] Wieland, H., and Rosenthal, C., *Ann. Chem.*, **554**, 241 (1943).

In a third experiment non-isotopic acetoacetate and succinate were used with isotopic bicarbonate. The fumaric acid isolated contained a normal concentration of C^{13}, indicating that, under the conditions of this experiment, CO_2 is not assimilated by kidney homogenates.

The results[3] of the first two experiments are shown in the accompanying tabulation.

The isolated fumaric and α-ketoglutaric acids contained excesses of C^{13} in an order of magnitude which would be expected if the acids of the tricarboxylic acid cycle are a major pathway of acetoacetate oxidation. A preliminary experiment indicates that the fumaric acid contained excess isotope only in the carboxyl carbons.

The α-ketoglutaric acid semicarbazone was quantitatively oxidized by acid permanganate into $2CO_2$ and succinic acid. The carboxyl carbon proximal to the keto group of α-ketoglutaric acid contained 0.24 atom per cent excess C^{13} and the succinic acid contained 0.59. Should all of the isotope of the succinic acid fraction prove to be located in only one position in the molecule (*i.e.* in the carboxyl group which was distal to the keto group of α-ketoglutaric acid), there would be 2.36 atoms per cent excess C^{13} in this position. Were citric acid an intermediate in acetoacetate oxidation, as suggested by Breusch and by Wieland and Rosenthal, the 2 carboxyl carbon atoms of α-ketoglutarate should have contained equal concentrations of C^{13}.

The oxidation of acetoacetate may occur by a mechanism similar to that suggested by Wood *et al.*[4] for pyruvate. In such a schema *cis*-aconitic acid would be the condensation product of oxalacetic acid and a 2-carbon intermediate common to acetoacetate and pyruvate metabolism. The concentration of C^{13} in the carboxyl carbon proximal to the keto group of α-ketoglutaric acid is small when compared with the isotope content of the remaining carbons and might result from a reversible side reaction between *cis*-aconitate and citrate.

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Received for publication, December 27, 1944

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[3] We wish to express our appreciation to Dr. Sidney Weinhouse and the Catalytic Development Corporation for the isotopic analyses reported

[4] Wood, H. C., Werkman, C H., Hemingway, A , and Nier, A O , *J Biol Chem.*, **139**, 483 (1941).

SOME BIOLOGICAL REACTIONS OF ACETIC ACID

Sirs:

The ability of the animal organism to synthesize acetyl groups and rapidly oxidize acetic acid raises the question as to the mechanism by which acetate is metabolized. It has been suggested that acetate or acetoacetate can enter the "citric acid" cycle by condensation with oxalacetate.[1] Citric acid formation from acetate and oxalacetate has been observed in yeast[2] and from acetoacetate and oxalacetate in kidney.[3] The intermediates of the "citric acid" cycle, ketoglutarate, oxalacetate, etc., cannot be isolated from the intact animal but glutamic and aspartic acids, which are in biological equilibrium with these keto acids, can be isolated from protein. We have fed labeled acetate containing 19.6 atom per cent excess C^{13} in the carboxyl group to both rats and mice and have found the liver dicarboxylic amino acids to contain C^{13}. Degradation by ninhydrin or chloramine-T of the glutamic acid shows that not all of the C^{13} is contained in the α-carboxyl group. The data are shown in the table; similar, though lower, values were found in the carcasses. While some of the C^{13} could have been introduced by CO_2 fixation, this mechanism cannot account for the C^{13} distribution we find. The findings indicate that a pathway exists by which acetate is utilized to form intermediates of the carbohydrate metabolism. As the tissue proteins have a high content of glutamic and aspartic acids, and as large quantities of ketoglutaric acid and oxalacetic acid arise from protein and carbohydrate metabolism, high C^{13} concentrations cannot be expected in the dicarbox-

C^{13} Concentrations in Liver Constituents in Atom Per Cent Excess

	Rats (fed labeled acetate for 3 days)	Mice (fed labeled acetate for 8 days)
Glutamic acid	0.035	0.080
α-Carboxyl of glutamic acid	0.041	0.096
Aspartic acid	0.023	0.038
Glycogen	0.020	0.038
Respiratory CO_2		0.066
Amidine carbon of arginine		0.080
Urinary urea	0.035	

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[1] Krebs, A., in Nord, F. F., and Werkman, C. H., Advances in enzymology and related subjects, New York, **2**, 191 (1942).

[2] Sonderhoff, R., and Thomas, H., *Ann. Chem.*, **530**, 195 (1937).

[3] Wieland, H., and Rosenthal, C., *Ann. Chem*, **554**, 241 (1943).

ylic amino acids, even if all the acetate were utilized in the formation of these compounds.

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KETONE BODY FORMATION FROM ACETATE IN KIDNEY, WITH ISOTOPIC CARBON AS A TRACER

Sirs:

Following the demonstration of the intermediary formation of acetyl groups in the conversion of fatty acids to ketone bodies by liver,[1] we were interested in ascertaining whether ketone bodies are formed by other tissues that metabolize acetate. Although acetate is oxidized rapidly by rat kidney slices without the accumulation of ketone bodies,[2] the possibility remained that they are formed but are further metabolized too rapidly to permit their detection by ordinary means. To test this possibility we carried out the following "isotope dilution" experiment. A mixture of isotopically labeled acetate and normal, non-isotopic acetoacetate was incubated with kidney slices. The amount of acetoacetate added was sufficient to allow the recovery of an appreciable quantity at the end of the incubation period. Under these circumstances any isotopic acetoacetate formed from acetate should mix with the normal acetoacetate; thus, the presence of excess isotope in the recovered acetoacetate would constitute proof of its formation from acetate.

Utilizing this procedure, we have now obtained definite evidence of the condensation of acetate to acetoacetate in kidney. The results of a typical experiment (one of three thus far carried out) are described below.

A mixture of 0.545 mM of sodium acetate, having an excess of 8.32 atom per cent C^{13} in the carboxyl carbon, and 0.297 mM of sodium acetoacetate, in 60 ml. of a Ringer-phosphate solution, was shaken in oxygen for 2 hours at 38° with 2.44 gm. of slices of whole rat kidney. There were recovered 0.453 mM of acetic acid and 0.140 mM of acetoacetate. The latter was decarboxylated to acetone and CO_2. The acetone had a C^{13} excess of 0.27 per cent. Iodoform, isolated by degradation of the acetone, had only the normal C^{13} concentration, indicating that the excess C^{13} was confined to the carbonyl group of acetone (the β-carbon of acetoacetate). Hence the C^{13} content of the β-carbon was $0.27 \times 3 = 0.81$ per cent. The CO_2 from the carboxyl group of acetoacetate had 0.82 per cent C^{13} excess.

There was also formed 0.071 mM of hydroxybutyrate which, on oxidation with dichromate, yielded acetone with 0.20 per cent C^{13} excess, representing 0.60 per cent in the β-carbon atom.

A rough calculation shows that of the total isotopic acetate utilized at least 55 per cent was transformed to ketone bodies. The equal distribution of the excess isotope between the carboxyl and β-carbon atoms of acetoacetate and the absence of C^{13} from the α- and γ-carbon atoms indicate that the ketone bodies arise in kidney by the same mechanism as in liver; *i.e.*, by coupling of acetyl groups.

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[1] Weinhouse, S., Medes, G., and Floyd, N. F., *J. Biol. Chem.*, **155**, 143 (1944).

[2] Unpublished observation.

Evidently ketone body formation is not exclusively a liver function, as is widely believed, but may occur generally in tissues which metabolize acetate.

We express our appreciation for the cooperation of Professor H. C. Urey and the Houdry Process Corporation of Marcus Hook, Pennsylvania.

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EFFECT OF NUCLEATES ON THE RATE OF METHYLENE BLUE DECOLORIZATION IN TISSUE EXTRACTS

Sirs:

Aqueous tissue extracts, containing reducing systems, possess the capacity of decolorizing methylene blue under anaerobic conditions. We have observed that when sodium yeast nucleate is added to such extracts the decolorization rate is slightly decreased; when sodium thymus nucleate is added, this rate is very considerably decreased. The extent of this decrease in rate is proportional to the amount of nucleate added. Addition of xanthine results in an increase in decolorization rate which appears to be very nearly the same whether nucleate is present or not. The percentage increase in rate on addition of substrate, however, is greatest in the presence of thymus nucleate. The fact that the activity of xanthine dehydrogenase is independent of the presence of nucleate indicates that there is no effect of the nucleate on the dye. The results of a typical experiment are given in the table.

Concentration of methylene blue 1.24×10^{-3} M, of the nucleates 1.0 per cent, of xanthine 1.6×10^{-3} M, each cc. of the liver extract equivalent to 300 mg of tissue; temperature 24–26°; anaerobic conditions throughout.

Mixture	Decolorization rate
	min.$^{-1} \times 10^3$
2 cc. liver extract + 1 cc. H_2O + 1 cc. H_2O + 1 cc. methylene blue	50
2 cc. liver extract + 1 cc. H_2O + 1 cc. xanthine + 1 cc. methylene blue	83
2 cc. liver extract + 1 cc. yeast nucleate + 1 cc. H_2O + 1 cc. methylene blue	40
2 cc liver extract + 1 cc. yeast nucleate + 1 cc. xanthine + 1 cc. methylene blue	71
2 cc liver extract + 1 cc thymus nucleate + 1 cc. H_2O + 1 cc. methylene blue	7
2 cc liver extract + 1 cc thymus nucleate + 1 cc. xanthine + 1 cc. methylene blue	38

Analogous results to those in the table have been obtained with tissue extracts from other species, as well as with suspensions of rat heart with succinate. Nearly identical results are obtained with freshly mixed solutions and with mixtures which are allowed to stand until the viscosity of the thymus nucleate is reduced nearly to that of the extract (enzymatic depolymerization). Porphyrindin oxidation of sulfhydryl groups in the extract has no effect on the rate of decolorization of the dye.

It is possible that the thymus nucleate is reduced by certain of the tissue components, and the oxidation of the reduced nucleate by the dye is a relatively slow process. We have noted that highly polymerized thymus nucleate treated with hydrosulfite takes up oxygen more rapidly than the reducing agent alone. The autoxidation of riboflavin reduced by hydrosulfite in the presence of thymus nucleate is appreciably delayed. Our results suggest that thymus type nucleates participate in the regulation of certain oxidation-reduction processes.

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